HIV-associated Cryptococcal Meningitis in sub-Saharan Africa: Factors affecting short and long-term survival

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Abstract

Cryptococcal meningitis (CM) is a wide-spread, yet under-recognized, fungal opportunistic infection occurring primarily among people living with advanced HIV/AIDS. While vast advances in understanding the pathogenesis and treatment options for CM have reduced mortality, major gaps remain in understanding factors that contribute to mortality rates of 12-20% in the first two weeks. The intent of this dissertation was to contribute towards the efforts to address these gaps and provide evidence that could further improve short and long-term recovery from HIV-associated CM in sub-Saharan Africa.

The first paper was focused on mortality in the days after CM diagnosis and understanding whether lumbar punctures (LPs) to reduce intracranial pressure also reduce mortality. Raised intracranial pressure is common in CM patients and contributes to many of the disease's signs and symptoms. Two hundred forty-eight HIV-positive, CM patients from Uganda and South Africa were evaluated for the occurrence of therapeutic LPs and mortality within 11 days of CM diagnosis. Analysis was conducted using a marginal structure model, with time-varying exposure. The results suggest that undergoing at least one repeat LP reduced 11-day mortality by 69% (95% CI: 18% to 88%), adjusted for heart rate, CSF fungal burden and altered mental status. This beneficial effect was independent of the baseline CSF opening pressure, demonstrating that increases in intracranial pressure may be common among all CM patients and that all patients may benefit from an additional LP during initial therapy.

The second paper was aimed at investigating baseline demographic and clinical features predictive of CM treatment success. The recommended initial treatment regimen for CM rapidly reduces infection; however, nearly 50% of CM patients will continue to have viable fungus in their central nervous system at the end of therapy. Being able to predict patient

outcomes has many advantages including optimizing treatment for each patient and reducing drug toxicities. One hundred seventy-seven HIV-positive, CM patients undergoing 2 weeks of antifungal treatment, including amphotericin B and fluconazole, were evaluated for sterility of a CSF culture by the end of therapy. The baseline CSF quantitative fungal burden was a strong and practical predictor of achieving CSF sterility, along with the rate of fungal clearance over the first week of treatment, and the baseline hemoglobin. Information on the baseline burden of infection could be used to tailor the duration of treatment, thus avoiding unnecessary toxicity and treatment costs for individuals with a lower burden of infection and potentially shifting resources to allow for more aggressive treatment of high-risk patients.

The third paper aimed to understand the consequence of residual fungal infection at the end of initial antifungal therapy in terms of mortality in the first weeks and months after 2-week therapy ends. Among 154 HIV-positive individuals surviving the initial 2-week phase of therapy, there was no evidence that either the presence or the amount of residual cryptococcal infection in the CSF had an association with mortality in the following 3 weeks or 6 months. It is possible that a higher dose of fluconazole used at the end of amphotericin therapy in the present cohort may have contributed to the lack of association.

The objective of this dissertation was to expand the understanding of CM treatment and recovery in resource-limited settings. The results suggest that additional benefits could be gained from the use of therapeutic lumbar punctures during the acute phase of treatment, the possibility of customizing therapy to further reduce treatment toxicities, and, finally, describing the relationship between residual infection and CM mortality with indirect support for higher doses of fluconazole during subsequent phases of CM therapy.

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Chapter 1

Introduction

1.1 Cryptococcus neoformans

Cryptococcus neoformans is a species in the phylum basidiomycota in the fungal kingdom. Other fungi in this phylum are more commonly known than *Cryptococcus spp.*, including portabello mushrooms and toadstools. The Cryptococcus genus contains two species of relevance to human health, *C. neoformans* and *C. gattii*. These two species are further divided into four serotypes, A, B, C, and D. Once classified as *C. neoformans var. gattii*, *C. gattii* is now its own species of Crytococcus. It comprises both serotypes B and C, and has been associated with outbreaks of meningitis in the Pacific-Northwest region of the United States and southwestern Canada. Two other varieties of *C. neoformans* are known, *C. neoformans var. grubii* and *C. neoformans var. neoformans*. These varieties are associated with serotypes A and D, respectively. As serotype A, and thus *C. neoformans var. grubii*, is responsible for the vast majority of clinical illness in the world, further discussion is focused on this particular variety of *C. neoformans* [1].

C. neoformans is found in many environments throughout the world. Historically associated with bird droppings, pigeon guano in particular, the environmental home of *C.*

neoformans also includes soil and numerous types of wood and tree bark. *C. neoformans* appears as a white or cream colored, opaque colony on laboratory media and clinical tissue samples. Its macro-level structure is composed of a cell body and a characteristic capsule and is between 5 and 10 micrometers in diameter, or about the size of a human red blood cell. The capsule is made up of polysaccharides and functions to protect cells from environmental desiccation and predation from other microorganisms [2]. Cryptococcal cells can shed or change their capsule spontaneously or in response to stressors [3]. The capsule can also enlarge over the life of the fungus resulting in cells with total diameters up to 50-100 micrometers, or nearly the width of a piece of paper [4].

C. neoformans has adapted additional features in order to survive environmental stresses, most notably melanin production. Melanin is a brown-black pigment and by-product of enzymatic conversion of surrounding substrates, including cathecolamines. Melanin protects cryptococcal cells from oxidizing agents, provides tolerance to hot and cold temperatures, and protects against ionizing radiation. In fact, many melanizing fungi, including *Cryptococcus spp.*, have been found to grow in the nuclear reactors at Chernobyl and on the exterior of the International Space Station [2, 5].

1.1.1 Interactions with humans

Early in life, most people are exposed to *C. neoformans* through inhalation of the small fungal cells kicked up in dust or dirt. Unlike many other microbial human pathogens, *C. neoformans* does not require a host for replication and survival. As such, human interaction with *C. neoformans* is coincidental and humans are dead-end hosts. Few studies have been done to elucidate the natural history and full extent of exposure to *C. neoformans*. However, available data suggests that by the age of 10 years, more than 60% of children in the urban setting of the Bronx, New York, 22% in rural New York State, and 18% in Manila, Philippines had serological evidence of prior exposure or subclinical infection with

C. neoformans [6, 7].

Both the cryptococcal capsule and the production of melanin, which support environmental survival, are also known virulence factors and support survival in the host. This is supported by animal experiments with unencapsulated strains of *C. neoformans* that show no virulence [2]. Additionally, nearly every clinical isolate from humans has been encapsulated, thus further supporting the role of the capsule in virulence [1]. The capsule is known to help cryptococcal cells evade the immune system in several notable ways; the capsule can enlarge enough to mechanically inhibit phagocytosis and the main material of the capsule, glucuronoxylomannan (GXM), is anti-phagocytic, inhibits antibody production against the capsule, and is an effective inducer of the complement system - perhaps so much so that complement can be exhausted [8, 9]. Melanin production additionally aids in resisting oxidative killing and provides tolerance to the warmth of the human environment. Being a facultative intracellular pathogen, the capsule of *C. neoformans* is needed for survival within a macrophage and melanin production additionally protects against the acidic environment of the phagolysosome [10].

1.1.2 Human immune response

Most people can effectively halt fungal replication in the lungs and either clear the infection or relegate *C. neoformans* to latent infection, such that healthy individuals rarely succumb to disease. Alveolar macrophages are the primary immune cell encountered by *C. neoformans* during initial lung infection. Attack and control of cryptococcal cells by these macrophages, and assisting cell-mediated immune responses, typically occurs swiftly and before fungal virulence factors can inhibit immune activity allowing cryptococcal cells can replicate [1]. In the absence of a robust cellular immune response, cryptococcal cells can proliferate in the lung and disseminate throughout the body.

While all body organs are susceptible to invasion, disseminated infection most commonly involves the central nervous system and clinically manifests as meningitis [11–13]. Invasion of the meninges likely occurs through direct passage of cryptococcal cells across the blood-brain barrier or transport across the barrier within an infected macrophage [14]. Once across the blood-brain barrier, its presence elicits inflammation and edema. Furthermore, the release of the polysaccharide capsule results in obstruction of crucial outflows for cerebrospinal fluid (CSF), resulting in increases in intracranial pressure that may contribute to the morbidity and mortality seen in cryptococcal meningitis [15, 16].

A cell-mediated immune response, rather than an antibody-mediated response, seems most critical for control of cryptococcosis, considering that antibody responses to *C. neoformans* are non-sterilizing and that most immune deficiencies that increase the risk of cryptococcal disease are deficiencies of cell-mediated responses [1]. Immunosuppressions from hematologic malignancies, steroid treatments, treatments associated with organ transplantation, diabetes, and HIV-infection have all been associated with invasive cryptococcal disease. It is currently unknown whether disseminated disease following immunosuppression is from a new exposure to *C. neoformans* or a reactivation of latent infection.

1.2 Human Immunodeficiency Virus

1.2.1 Epidemiology

Worldwide, infection with HIV is the most significant cause of immunosuppression affecting roughly 33.3 million people in 2009 [17]. Roughly two-thirds of people living with HIV/AIDS, an estimated 22.5 million people, are living in sub-Saharan Africa. Prevalence rates in Eastern and Southern Africa indicate that many countries in sub-Saharan Africa still experience a generalized HIV epidemic. While HIV epidemics in the Americas and Europe are largely driven by homosexual transmission and injection drug use, the African

HIV/AIDS epidemic is largely driven by heterosexual transmission.

In 2009, it was estimated that 1.8 million new HIV infections occurred in sub-Saharan Africa, accounting for 69% of all new infections globally [17]. The good news is that these estimated incidence rates were stable or have declined in comparison to estimates from 2001, and an 18% decrease in new infections was seen in the region overall [17]. The number of AIDS deaths has also declined over the past 5 years, but the unfortunate story is that an unimaginable number of people continue to die from AIDS each year. In 2009, an estimated 1.3 million Africans died of AIDS [17].

HIV is a retrovirus transmitted between humans through contact with blood and bodily fluids. The virus causes immunologic decay through depletion of CD4-positive lymphocytes, one of the virus's target cells. HIV has this effect by directly killing the CD4 cell, inducing apoptosis, or allowing immunological clearing of infected CD4 cells. CD4 cells are immensely important for activation of cell-mediated and humoral immunity, and over many years of uncontrolled HIV replication, the CD4 cells are reduced so much that the human host becomes susceptible to infections. The condition of AIDS occurs when the immune system is so depleted (CD4 $<$ 200 cells/μL) that it is incapable of protecting against infection.

With reduction in the capacity of the immune system, HIV-positive individuals progressively become more susceptible to diseases commonly controlled in healthy individuals. Worldwide, opportunistic infections (OIs) such as Kaposis sarcoma, pneumocystis pneumonia, oral candidiasis, toxoplasmosis, tuberculosis, and cryptococcal meningitis, which are routinely controlled in immunocompetent individuals, are commonly seen in individuals infected with HIV/AIDS.

1.2.2 HIV and cryptococcal meningitis

Before the HIV epidemic, cryptococcosis and cryptococcal meningitis (CM) were rare. Since then, CM is seen in HIV-positive individuals with advanced immunosuppression, and the majority of the cryptococcal literature has focused on HIV-associated cryptococcosis [18–22]. It is estimated that each year up to 1 million cases of CM may occur in people living with HIV/AIDS [23].

Cryptococcal meningitis in well-resourced areas In the early 1990s the annual incidence of invasive cryptococcal disease among HIV-positive persons in the United States was estimated at 17-66 per 1,000 [13, 20]. Even with the best available antifungal treatments, the 2-week case-fatality rate for CM was 10-19% in the late 1980s and 1990s [13,24,25]. With the advent of highly active antiretroviral therapy (HAART) to control the progression of HIV infection and restore immune function, the incidence of CM has rapidly declined in the US, and throughout the developed world [26]. The yearly incidence is now estimated to be 7.8 and 0.5 per 1,000 in North America and Europe, respectively, with a low case-fatality rate, assumed to be 9% in the first 90 day [23, 27].

Cryptococcal meningitis in less-resourced settings Unfortunately, the enormity of the HIV epidemic and limited access to HAART in developing parts of the world has meant that the 22.5 million HIV-positive individuals in sub-Saharan Africa still experience a high incidence of CM. A 2004 population-survey in Gauteng Province, South Africa estimated an incidence rate of 14 cases per 1,000 persons with AIDS [28]. Other estimates throughout sub-Saharan Africa suggest that incident CM could occur in 3.2% of the HIV-positive population annually, resulting in 720,000 cases in 2006 [23]. From studies conducted in research settings, the best 2-week mortality rate may be 12-20% with the current standard treatments in sub-Saharan Africa [29–31]. However, realities limit the

availability of drugs and supplies to treat CM such that case-fatality rates of 27-75% are observed and assumed to be much more representative of the impact of CM [28, 32–36]. Based on these latter case-fatality rates, approximately 504,000 deaths from CM occurred in sub-Saharan Africa in 2006 [23]. To put this in context, CM was estimated to be the fourth most common cause of death from infectious disease in sub-Saharan Africa in 2006, excluding HIV, and this estimate likely overshadowed mortality from tuberculosis [23].

Clinical aspects of cryptococcal meningitis Clinically, cryptococcal meningitis presents with headache, neck pain, fever, and, sometimes, altered consciousness. Occasionally, additional symptoms of meningeal involvement, including blurry vision, changes in mood or behavior, and confusion, are present. Diagnosis of CM is made by detection of viable *C. neoformans* on CSF culture or by detection of the cryptococcal capsule in the CSF via immunoassay or microscopy staining with India ink. The capsular antigen can also be detected in peripheral blood, and immunoassays of blood samples are sometimes used for diagnosis of CM or extraneural cryptococcal infection.

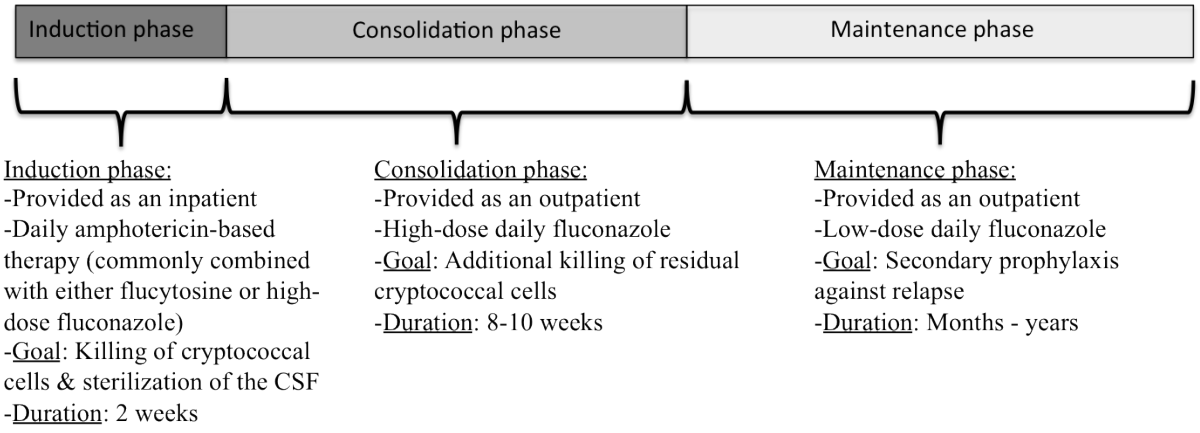


Figure 1.1: Timing and content of the recommended antifungal treatment strategy for cryptococcal meningitis [37].

International treatment guidelines recommend prompt treatment after CM diagnosis. Treatment occurs in three phases: 1) induction, 2) consolidation, and 3) maintenance (figure 1.1) [37,38]. Induction therapy is aimed at rapidly killing *C. neoformans* and sterilizing the CSF. Recommended induction therapy consists of 2 weeks of daily intravenous amphotericin B in combination with another antifungal, either flucytosine or fluconazole. While flucytosine is the most effective choice, it is not licensed in sub-Saharan Africa and fluconazole is often the only available adjunctive therapy outside of research protocols. Consolidation therapy, which serves to continue controlling cryptococcal replication, consists of 8-10 weeks of daily oral fluconazole. A lower dose of daily fluconazole is used for the final maintenance phase of treatment. This later maintenance phase is meant as secondary prophylaxis against cryptococcal relapse and is recommended to continue until immune restoration (CD4 count $>$ 200 cells/μL) is achieved.

Currently, 60-70% of HIV-positive CM patients are found to have negative CSF cultures for *C. neoformans* at the end of induction therapy [39, 40]. The probability that an individual will reach CSF sterility by the end of induction therapy seems largely dependent on the baseline fungal burden, the extent of dissemination of infection, and the inflammatory nature of the initial immune response [25, 39–43], though few studies have been conducted outside of resource-rich settings.

1.3 Long-term sequelae from cryptococcal meningitis

As mentioned above, fatal CM is a common occurrence in sub-Saharan Africa, with contemporary estimates of 40-60% mortality within 6 months of diagnosis [30, 35, 44]. As may be anticipated, much of this mortality occurs soon after hospital admission and CM diagnosis, and roughly 20% of individuals may die during induction phase of therapy (figures 1.2 and 1.3). Most of the remaining mortality occurs within the first month after the end

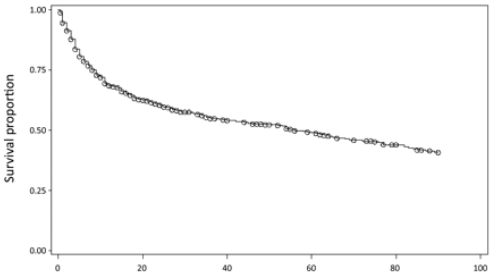


Figure 1.2: Kaplan-Meier survival probabilities for cryptococcal meningitis, in days from cryptococcal meningitis diagnosis in HIV-positive individuals from Gautang, South Africa [35].

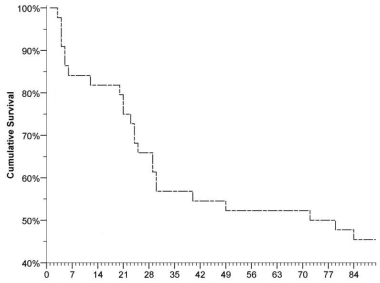


Figure 1.3: Kaplan-Meier survival probabilities for cryptococcal meningitis, in days from cryptococcal meningitis diagnosis in HIV-positive individuals from Kampala, Uganda [30].

of induction therapy.

Beyond the first month, continued sequelae from CM and advanced HIV, such as re-hospitalization from CM relapse or CM-related immune reconstitution inflammatory syndrome (CM-IRIS) after initiation of ART, begin to occur. CM-IRIS is a paradoxical, dysregulated inflammatory reaction that occurs after the immune system has recovered from severe immunosuppression. Re-hospitalization, for any number of reasons, after initial CM occurs in roughly one-third of patients (unpublished data). In a study of long-term outcomes in South Africa, 56% of deaths were associated with readmissions after induction therapy, further highlighting the impact that long-term sequelae have on overall CM mortality [35].

Laboratory-confirmed relapse of CM is strongly related to adherence to the consolidation and maintenance phases of antifungal therapy [25, 45]. In Cape Town, South Africa, roughly 1 case per month (or 20% of all CM-associated admissions) was a case of CM relapse [45, 46]. Among these relapse cases, it was found that nearly half, 43%, were due to a lack of secondary fluconazole prophylaxis [45]. Additionally, the incidence of CM-IRIS in sub-Saharan Africa contributes to the overall morbidity and mortality after the induction phase of antifungal therapy [47].

1.4 Critical aspects of cryptococcal meningitis

1.4.1 Intracranial pressure

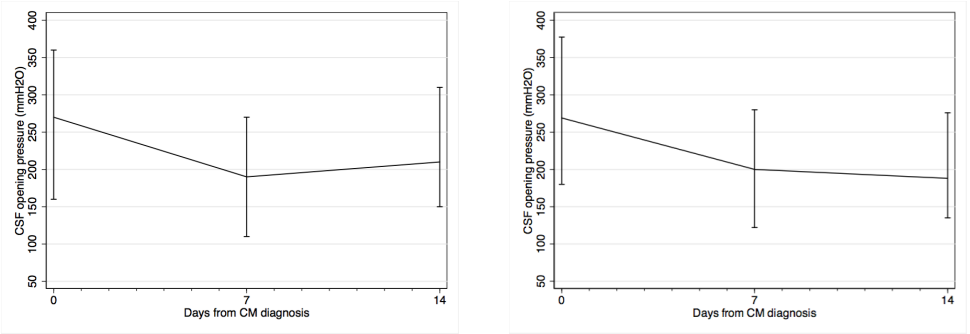
Understanding of the pathogenesis of infection with *C. neoformans* and the severity of cryptococcal disease progression with HIV infection has led to vast improvements in treatment in both settings of many and few resources. One particular feature of CM treatment, the management of raised intracranial pressure, warrants special attention for this dissertation.

The detriment of raised intracranial pressure (ICP) in CM was initially noted by case reports of high mortality despite effective treatment [15, 48, 49]. Subsequent reports and observational studies indicated that raised ICP is a common feature of CM, occurring in 60-75% of patients at the time of diagnosis, and current treatment guidelines stress the importance of management of raised ICP not only to reduce neurological, visual, and auditory sequelae but also to reduce mortality [24, 37, 38, 50–52].

Intracranial pressure is essential to maintaining flow of cerebrospinal fluid (CSF) around the brain. The CSF acts to keep the brain buoyant, remove waste from neuronal metabolism, and maintain a stable environment for neuronal function. The rate of CSF absorption and, by extension the rate of CSF flow through the ventricles and subarachnoid space, depends on the pressure in these spaces. The ICP, as measured by the opening pressure of the CSF during a lumbar puncture, is normally 5-15 mmHg or 60-180 mmH_2O [53]. It acts together with the systemic arterial pressure to maintain adequate perfusion of the brain with blood and CSF. The cerebral perfusion pressure (CPP) is described as the difference between the mean arterial pressure (MAP, a proportionality between diastolic and systolic blood pressure) and the intracranial pressure: CPP = MAP - ICP [53]. Intracranial pressure rises in response to changes in the volume of the brain, as with edema, hemorrhage, or tumor growth. Pressures may also increase if CSF absorption is slowed, as is likely the case with cryptococcal meningitis. Pressures beyond 250 mmH_2O are considered abnormally high in

CM patients.

Slow or short-term increases in ICP can be compensated with increases in arterial pressure without overt symptoms [54]. However, larger and longer periods of raised ICP require a larger compensation by systemic vasoconstriction, which can result in clinical symptoms such as confusion, restlessness, drowsiness, and changes in breathing. As the ICP nears the level of MAP, cerebral perfusion is greatly reduced causing hypoxia and clinical signs including decreased levels of consciousness. Perfusion of the brain ceases when the ICP equals the MAP. To avoid detrimental levels of ICP, treatment guidelines for CM suggest measurement of ICP at the time of diagnosis and repeat lumbar punctures in those with initially high pressure when symptoms of raised ICP are still present [38]. While the exact mechanism of increased ICP with CM is unknown, it may be related to active replication of *C. neoformans* and the immune response to that replication. Collections of viable fungal cells and immune cells crowd around the arachnoid villi, thus blocking CSF outflow [15, 16, 51, 55]. Intracranial pressure can flunctuate in CM patients over the course of induction therapy with high pressures at diagnosis, possibly reflecting the fungal load in



(a) (b)

Figure 1.4: Median CSF opening pressures over treatment for cryptococcal meningitis among (a) 75 HIV-positive patients with cryptococcal meningitis in South Africa and Thailand [50], and (b) 152 HIV-positive patients with cryptococcal meningitis in from Uganda and South Africa from the Cryptococcal Optimal ART Timing trial. Vertical bars depict the 25^{th} to the 75^{th} percentile range.

the central nervous system, and reduced pressures over the course of treatment as fungal burden declines (figure 1.4). Some patients may experience increases in pressure during the second week of therapy [50]. Raised ICP is typically managed in CM patients with cerebral shunts or by mechanical removal of CSF through repeated lumbar puncture or in-dwelling lumbar drains.

Increased ICP at hospital admission may have a strong impact on mortality risk during the first week of CM therapy. Fatal outcomes during the induction phase of therapy in CM patients have been associated with elevated CSF opening pressure in some observational studies and the authors of these studies point to increased vigilance to control ICP in order avert these deaths [25, 39, 51]. However, other studies have not found evidence of associational effects of raised ICP on short 2-week or long-term (10-week) mortality [30,50]. In a study of patients from South Africa and Thailand, frequent lumbar punctures were performed and may account for the lack of association between raised ICP and CM mortality [50]. Conversely, a smaller study from Uganda was unable to employ frequent serial lumbar punctures due to patient resistance to the procedure, and no evidence suggested that those with higher baseline CSF opening pressure had greater mortality during the 2 weeks of induction therapy [30]. This possibly suggests that the association between raised ICP and fatal CM is more complex than previously recognized. No studies have evaluated the association between high ICP and mortality in the first week following diagnosis, and no studies have rigorously evaluated the effect of lumbar punctures interventions on mortality.

1.5 Gaps in the literature

The literature on cryptococcal meningitis in HIV-positive Africans has grown in recent years and answered many important questions regarding the burden of CM [23], efficacy of

antifungal regimens [29, 31, 56–59], predictors of 2-week mortality [21, 30, 32, 34, 36, 60–65], and the pattern of outcomes after antifungal induction therapy [23, 30, 45, 63]. However, many questions remain, such as those addressed in this dissertation.

1.5.1 The role of intracranial pressure on CM mortality

Additional lumbar punctures to control intracranial pressure during the acute episode of CM are recommended by international treatment guidelines, yet few studies in sub-Saharan Africa have evaluated the role of raised ICP on acute mortality and those that have have found no evidence of an association with 2-week mortality [30, 44, 50, 51, 60]. Whether these null findings are due to limits of sample size, adequate pressure control practices, or a more nuanced association between ICP and mortality are both possible. As mentioned, the impact of ICP on survival from CM may be most evident among deaths occurring the first week after diagnosis. Thus, a current gap in the literature that this dissertation aims to address is whether those with more lumbar punctures to control ICP during the first week of induction therapy have improved survival compared to those without additional lumbar punctures. Addressing this question will shed further light on the role of ICP in contributing to mortality soon after CM diagnosis and may help to inform recommendations for supportive care.

1.5.2 Predictors of CSF sterility at the end of induction therapy

Given that a CM patient survives to complete antifungal induction therapy, perhaps 40% will continue to have a positive CSF fungal culture at the end of this period. Few studies in sub-Saharan Africa have assessed factors that predict who will achieve sterility and who may not [43]. While biologic risk factors related to the course of CM may not differ between patients living in the Western world and sub-Saharan Africa, risk factors dependent on hospital resources or severity of disease may be vastly different.

1.5.3 Impact of residual fungal burden on mortality after initial CM therapy

Severe CM, as marked by altered mental status and high baseline fungal load, is commonly known to be predictive of mortality within the first 2 weeks of CM diagnosis. But few risk factors for mortality after this 2-week period have been described in sub-Saharan Africa. Among the clinical factors that have been evaluated, not surprisingly, more severe disease (as indicated by seizures, altered mental status, or coma on admission), high baseline fungal burden, low weight, and lack of amphotericin-based antifungal treatment were found to be associated with shorter survival after the initial hospitalization [35, 44, 60]. These factors suggest that persistent infection could be related to mortality, as those with high baseline fungal burdens and inadequate therapy are more likely to have positive CSF cultures at the end of induction therapy. Because a good share of individuals continue to have viable *C. neoformans* in their CSF, it is important to understand what impact this persistent infection may have, particularly with regards to the high mortality that continues after induction therapy ends. What is known is that residual cryptococcal infection may increase the risk of subsequent CM relapse and CM-IRIS [25, 41, 47, 58, 66, 67]. Thus, the pattern of illness after an episode of CM suggests there is a detriment to incomplete sterilization of the CSF during amphotericin-based induction therapy. Few studies have remarked on the impact of residual fungal burden and mortality after CM induction therapy, and only one study has been conducted in sub-Saharan Africa [43] It is unclear whether residual fungal burden also contributes to the deaths that occur in the weeks and months following induction therapy.

1.6 Summary

This brief overview demonstrates the significant burden of cryptococcal meningitis among people living with HIV/AIDS in sub-Saharan Africa. It is estimated that roughly 720,000 cases of CM will be diagnosed each year and the annual mortality rate may exceed that of tuberculosis. Great advances in the treatment of CM have been made since the beginning of the HIV/AIDS epidemic, however sub-Saharan Africa is still plagued by limited resources such that patients with CM have delayed presentation and high case-fatality rates despite effective antifungal regimens. This dissertation aims to fill some of the gaps in understanding the causes and consequences of raised ICP and residual cryptococcal infection. By doing so, the results will help inform treatment recommendations and identify possible strategies that can continue to reduce the morbidity and mortality from CM in sub-Saharan Africa.

Chapter 2

Study Population

Two contiguous study populations are used throughout this dissertation. All chapters use data from the Cryptococcal Optimal ART Timing (COAT) Trial and Chapter 3 additionally includes data from an observational cohort started after COAT enrollment ended.

2.1 COAT trial

The Cryptococcal Optimal ART Timing (COAT) trial was designed to assess whether initiation of antiretroviral therapy (ART) during the second week of CM induction therapy was associated with improved 26-week survival compared to deferring ART initiation until 3 weeks after the end of CM induction therapy. Individuals eligible for COAT were HIV-positive, ART-naïve individuals, 18 years or older, diagnosed with a first episode of CM in tertiary referral hospitals in Kampala or Mbarara, Uganda or Cape Town, South Africa. Subjects were followed up for 46 weeks after the CM episode and observed for death, adverse events, and immune reconstitution inflammatory syndrome (IRIS). Treatment allocation was randomized within strata of individuals defined by the study site and presence of altered mental status at CM diagnosis. Written informed consent was received from all

study participants or from an identified surrogate if the participant was in an altered mental state. All participants who were enrolled under surrogate consent were re-consented for participation once the individuals regained normal mental status.

2.1.1 Clinical care and treatment

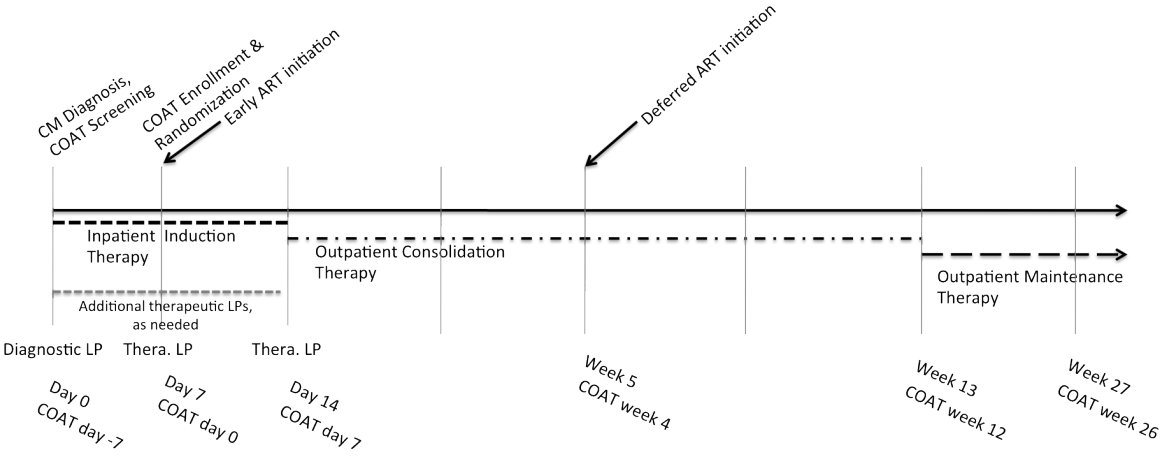


Figure 2.1: Timing of antifungal therapy, diagnostic lumbar puncture (LP), therapeutic (thera.) LPs, and relevant events in the COAT Trial.

At hospital presentation a screening physical exam and diagnostic lumbar puncture were conducted on all potential study subjects by the sites' clinical teams. A complete assessment of pre-existing conditions, prior AIDS defining illnesses, and the subject's mental status was conducted 3 days prior to randomization. Randomization occurred 7 to 11 days after CM diagnosis (figure 2.1). Clinical care and treatment of CM was standardized across sites with some modifications based on clinician judgment. The induction treatment regimen for CM consisted of 0.7-1.0 mg/kg/day amphtotericin B plus 800mg/day fluconazole for 2 weeks after diagnosis. Patients remained hospitalized throughout induction therapy

for observation and supportive care. Induction therapy was followed by the consolidation treatment phase, consisting of 3 additional weeks of 800mg fluconazole daily followed by 400mg fluconazole daily for 8 weeks. Thereafter, 200mg fluconazole daily was prescribed for maintenance therapy, which was given for 12 months and until the individual's CD4 cell count was at least 200 cells/μL for at least 6 months.

Protocol-specified lumbar punctures were also conducted at the time of randomization and again at study day 7 to monitor fungal clearance. Additional LPs could occur during induction therapy when deemed necessary by the clinical team. In accordance with international treatment guidelines, additional therapeutic LPs were considered for patients who met the criteria for symptomatic raised ICP, including headache, fever, meningismus, cranial nerve deficits, visual changes, and behavior changes or when prior opening pressures were greater than 250 mmH_2O [37]. Verbal consent, from the subject or a surrogate, if the subject was in an altered mental state, was required for all LPs. All LPs after the first diagnostic LP were considered therapeutic LPs.

Clinical exams, including vital signs, and assessment of drug adherence and new symptoms occurred at randomization, every 2-3 days during the second week of hospitalization, as an outpatient at study week 2, and every 2-4 weeks thereafter until 12 months had elapsed or the subject had died, withdrew consent, or was lost-to-follow-up.

2.1.2 Laboratory assessments

The diagnostic lumbar puncture, occurring at the time of hospital admission, included measurement of CSF opening and closing pressure and analysis of CSF by fungal culture, India ink staining, cryptococcal antigen latex agglutination assay, acid-fast staining, as well as cell counts and protein analysis. Limited CSF data collection was required at each additional LP, but included CSF pressures, cell counts, protein analysis, and fungal cultures.

Hematologic parameters were assessed at nearly every visit. In brief, and particular to this dissertation, CD4 cell counts were measured at randomization, study day 7, week 4, week 8, week 12, and week 26. HIV viral loads were additionally measured at randomization, study week 4, week 12, and week 26. CSF and serum cytokines were measured at screening, randomization, study day 7, and week 2.

2.1.3 Trial endpoints

The primary endpoint of the COAT trial was 26-week survival. Secondary endpoints included 46-week survival, incidence of immune reconstitution inflammatory syndrome (IRIS), Karnofsky performance score, microbiologic clearance of CSF by 26 weeks, and virologic suppression by 26 weeks.

2.1.4 Trial recruitment and conduct

The COAT trial was to enroll 500 CM patients. However, after a second interim analysis of the COAT trial on April 27, 2012, the independent data safety and monitoring board found sufficient evidence of excess mortality in the early ART arm and halted further enrollment and randomization. At that time 177 individuals had been randomized, 88 to the early ART arm and 89 to the deferred ART arm. By site, this included 115 individuals from Kampala, 35 from Mbarara, and 27 from Cape Town.

2.2 Observational cohort

Following the end of COAT enrollment, patients seen at Mulago Hospital, in Kampala, Uganda, with suspected meningitis were screened for inclusion into an observational cohort to assess neurocognitive recovery after meningitis. Eligible individuals were HIV-positive and only those with diagnosed CM were included in the analysis for this dissertation. The

inclusion and exclusion criteria were identical to those used in the COAT trial. Protocol specified patient care was also identical to the COAT protocol, including CM treatment regimen, laboratory assessments, and management of intracranial pressure. Patients were initiated on ART 5 weeks after CM diagnosis. Patients screened for participation in the observational cohort from April 28, 2012 through December 2012 were considered for inclusion in this dissertation.

Chapter 3

Therapeutic Lumbar Punctures and Acute Mortality from Cryptococcal Meningitis

Introduction *Cryptococcus neoformans* is an opportunistic fungal pathogen responsible for as many as 1 million cases of cryptococcal meningitis (CM) and over 500,000 deaths worldwide each year, mostly among those with HIV/AIDS. Individuals with CM typically have high intracranial pressure as a result of blocked outflow of cerebrospinal fluid (CSF) in the central nervous system. Prior studies suggest an association between this raised pressure and CM mortality. As such, current treatment guidelines for CM recommend removal of CSF, by lumbar puncture (LP) or drain, to reduce intracranial pressure. However, the impact of lumbar punctures to reduce CM-related mortality has not been assessed. The aim of this analysis was to evaluate the effect of therapeutic lumbar punctures on mortality within 11 days of CM diagnosis.

Methods Two hundred forty-eight HIV-positive, ART-naïve individuals diagnosed with CM were eligible for inclusion in this analysis. All individuals had an LP at the time of CM diagnosis and subsequent LPs were recommended for those with CSF opening pressures greater than 250 mmH_2O or symptoms of raised pressure. All patients received CM therapy according to current standard of care. Therapeutic LPs were defined as an additional LP occurring at least one day after the diagnostic LP. A marginal structural model, based on pooled Poisson regression with inverse-probability weights, was used to assess the effect of therapeutic LPs on short-term mortality, up to 11 days after CM diagnosis. Multiple imputation was used to account for missing baseline covariates. Adjustments for baseline closing pressure, fungal burden, heart rate, mental status, and weight were considered. Results are presented in terms of rate ratios with corresponding 95% confidence intervals.

Results Of 248 individuals, 75 (30%) had at least one additional LP within 11 days of the diagnostic LP and were conducted among individuals with and without high CSF opening pressures at baseline. Those who received additional therapeutic LPs were more likely to have lower heart rates, lower mental status, higher CSF opening and closing pressure, higher CSF fungal burden, and higher body weight, compared to those who did not. Thirty-six (15%) individuals died within 11 days of diagnosis, 31 deaths occurred among those without any additional LPs, whereas only 5 deaths were among those with at least one additional LP. The crude, unweighted regression indicated a relative risk of mortality of 0.53 with at least one additional LP (95% confidence interval: 0.20, 1.37) compared to no therapeutic LP. With adjustment for possible confounding by heart rate, CSF fungal burden, and an indicator for low mental status, the weighted marginal structural model relative risk of mortality was 0.31 (95% confidence interval: 0.12, 0.82), indicating a significant reduction in acute mortality with at least one therapeutic LP. There was no evidence that this effect was impacted by baseline CSF opening pressure.

Conclusions This analysis suggests that, among all CM patients, regardless of baseline CSF opening pressure, there is a survival benefit to undergoing at least one additional LP beyond the diagnostic LP to reduce intracranial pressure. Further investigation of the use of therapeutic LPs is warranted to evaluate the possible benefit in larger groups of CM patients.

3.1 Introduction

Despite recognition of the burden of cryptococcal disease in the era of HIV/AIDS and advancements in treatment recommendations and protocols, acute mortality from cryptococcal meningitis (CM) in resource-limited settings remains high. Contemporary mortality estimates, from clinical trials and cohorts, suggest that under the best conditions 12-20% of HIV-positive individuals in sub-Saharan Africa diagnosed with CM will die within two weeks of diagnosis [29–31, 43, 44]; however, higher rates may be more realistic outside of research settings [23,28,32–34,36]. The cause of such high mortality may stem from, among other things, late presentation to care and resource limitations that delay diagnosis.

One important complication of late disease presentation is elevated intracranial pressure (ICP). Many reports and cohorts have demonstrated that raised intracranial pressure, namely a cerebrospinal fluid (CSF) opening pressure of 250 mmH_2O or greater, is a detrimental consequence of CM. Cohorts of CM patients observed early during the AIDS epidemic in the US found a high proportion of deaths related to elevated ICP during the first two weeks of antifungal therapy [25, 51, 68].

Most instances of raised ICP in CM patients occur at the time of diagnosis and during the induction phase, or first two weeks, of antifungal therapy. Raised ICP can lead to changes in mental status, headache, loss of vision and hearing, or death. Therefore, aggressive management of ICP is included in national and international CM treatment

guidelines. Current guidelines recommend measurement of CSF opening pressure during the first, diagnostic lumbar puncture with reduction of pressures at or above 250 mmH_2O by 50% or to a closing pressure of 200 mmH_2O or less. In patients with pressures persisting above 250 mmH_2O and with symptoms of raised ICP, further therapeutic lumbar punctures should follow daily until the pressure and symptoms have normalized for at least 2 days [37, 38]. While symptoms of raised ICP can be subtle or absent in some patients, headache and visual or auditory changes are commonly seen in CM patients with elevated ICP, and signs and symptoms such as changes in mental state and papilledema may be indicative of extremely high pressures [50, 51].

Tools for managing intracranial pressure include mechanical removal of CSF or surgical placement of a ventricular shunt. Due to resource limitations and concerns for secondary infection, repeated lumbar punctures are commonly used in sub-Saharan Africa to manage ICP. In fact, the lack of association between baseline opening pressure and 2-week mortality seen in some cohorts has been attributed to good attention to and control of CSF opening pressure with serial LPs [50,69]. While studies have evaluated the effect of ICP on mortality from CM, only one small, feasibility trial has evaluated the effect of therapeutic LPs during acute CM [70]. This trial, which randomized 18 individuals to either daily LP or LPs on day 0, 7, and 14 of CM induction therapy, noted declines in CSF opening pressures in both arms but was not powered to evaluate the effect on mortality.

Much of the mortality thought to be attributable to raised ICP occurs in the first week of CM therapy [30, 35, 71]. Therefore, the objective of this paper was to evaluate the effect of controlling intracranial pressure on acute mortality using marginal structural models in an observational cohort of HIV-positive, ART-naïve, CM patients in South Africa and Uganda. The leading hypothesis is that at least one therapeutic LP after CM diagnosis will be related to a lower risk of mortality.

3.2 Methods

3.2.1 Study Population

This analysis is a sub-study of the Cryptococcal Optimal ART Timing (COAT) trial, which occurred from November 2010 to April 2012, and an observational cohort of CM patients that extended from April through December 2012. The COAT trial was designed to evaluate whether early ART initiation, one week after CM diagnosis, resulted in improved 26-week survival compared to deferring ART initiation until five weeks after CM diagnosis. Individuals with suspected meningitis were recruited for the COAT trial from 3 sites: Mulago Hospital/Infectious Diseases Institute in Kampala, Uganda; Mulago-Mbarara Joint AIDS Program and Mbarara University of Science and Technology in Mbarara, Uganda; and GF Jooste Hospital in Cape Town, South Africa. Recruitment into the observational cohort occurred at the Mulago Hospital/Infectious Diseases Institute in Kampala, Uganda. HIV-positive, ART-naïve individuals with CM were eligible for enrollment for either the trial or the cohort if they were at least 18 years old, receiving amphotericin-based CM treatment, willing to attend regular clinic visits, and provided informed consent. Individuals who were pregnant, breastfeeding, on immunosuppressive therapy, unable to take oral medication, had contraindications to study medications, had been on antifungal therapy for more than one week, or had a prior episode of CM were all excluded.

All subjects provided written informed consent to undergo an LP for diagnosis. Cryptococcal meningitis was diagnosed by a positive CSF cryptococcal culture or cryptococcal antigen (CRAG) latex agglutination assay. Treatment for CM consisted of 2 weeks of 0.7-1.0 mg/kg/day Amphotericin B plus 800 mg/day fluconazole followed by 3 weeks of 800 mg/day oral fluconazole, 8 weeks of 400 mg/day oral fluconazole, and 200 mg/day oral fluconazole for one year and until the individual's CD4 cell count was at least 200 cells/μL. Baseline clinical and laboratory features, including CSF parameters, were collected at the

time of CM diagnosis for all screened individuals. CD4 and HIV viral load measures were not collected until subjects were randomized into the COAT trial, thus these data are unavailable for all screened individuals.

Two hundred fifty-seven individuals were found to be eligible and were considered for inclusion in this analysis. Follow-up began the day after CM diagnosis in order to allow individuals the opportunity for exposure. Nine individuals were found to have died, been censored, or had a therapeutic LP on the same day they were screened for enrollment so were excluded from further analysis, leaving 248 individuals in the final cohort with 204 from the COAT trial and 44 from the observational cohort. Sensitivity analysis indicated that inclusion of these 9 individuals did not vary the conclusions of the final model (see Appendix A, section A.1). Observation ended at the time of death and individuals were censored at COAT randomization (between 7 and 11 days after CM diagnosis), or after 11 days, whichever occurred first. Randomization censored individuals because the COAT protocol indicated that an LP was to be done at randomization, thus the maximal observation time was 11 days after CM diagnosis.

Approval for the COAT trial was granted by the research ethic committees of: University of Minnesota, Makerere University, Mulago Hospital, Mbarara University, University of Cape Town, Uganda National Council of Science and Technology, South African Medicines Control Council, and NIH NIAID Clinical Science Review Committee (www.clinicaltrials.gov: NCT01075152). Approval for the observational cohort was provided by University of Minnesota, Makerere University, and Uganda National Council of Science and Technology. Approval for this analysis was additionally granted by the Institutional Review Board at the University of Minnesota.

3.2.2 Lumbar Punctures and CSF Parameters

Every subject received an initial LP for diagnostic purposes at the time of hospital admission. CSF opening pressures were measured at each LP with the use of a manometer. Additional lumbar punctures were recommended for those with CSF opening pressures greater than 250 mmH_2O or symptoms of raised pressure, according to guidelines set out by the Infectious Diseases Society of America [37]. Subjects could receive multiple therapeutic LPs during the period of CM therapy, at the discretion of the attending clinician, but exposure was defined as at least one therapeutic LP. Verbal informed consent was provided before all therapeutic LPs.

CSF fungal cultures were conducted in a microbiology lab, approved by the US National Institutes of Health, at each study site. CSF was plated onto Sabouraud's dextrose agar and incubated at 30 ^{o}C to allow fungal growth. Cultures were qualitatively considered positive if *C. neoformans* was detected and negative in the absence of fungal growth after 14 days of incubation. Quantification of *C. neoformans* was conducted using serial dilutions of CSF and counting colony-forming units (CFUs) of *C. neoformans* seen on the most diluted plate where growth was observed [29].

3.2.3 Statistical Analysis

An individual's status of having at least one therapeutic LP was not defined at the start of the cohort follow-up. As such, the effect of therapeutic LPs was assessed using a model with time-varying exposure. Individuals contributed person-time to the no therapeutic LP group after their diagnostic LP and until they received a therapeutic LP, died, or were censored. Once a therapeutic LP was performed, individuals then contributed person-time to the group who received at least one therapeutic LP. Crude mortality rates were calculated for person-time before a therapeutic LP as well as for person-time observed

after a first therapeutic LP. Chi-square and Wilcoxon ranksum tests were used to evaluate differences in baseline factors by eventual LP and vital status.

The primary objective was to quantify the average effect of therapeutic LP on acute mortality after CM diagnosis. The outcome of interest was all-cause mortality within 11 days of diagnosis. To account for possible confounding factors, a pooled Poisson regression framework with time-varying intercept was used to construct a marginal structural model with inverse probability weights. A log link with a Poisson distribution was used to estimate the relative risk [72, 73]. Robust standard errors, using generalized estimating equations, were calculated for the marginal structural model to account for intra-subject correlation induced by the weighting scheme [74] and to adjust the model for estimation of the relative risk [72]. Unweighted estimates of the effect were constructed from a similar pooled Poisson regression framework using a time-varying intercept, Poisson distribution, log link, and robust standard errors to estimate the relative risk of mortality and included possible confounders as additional covariates in the model.

For calculating exposure and censoring weights, logistic regression models were used to estimate both the probability of having at least one therapeutic LP during follow-up (exposure) and the probability of being censored over the observation time. Stabilized weights for exposure were estimated as the unconditional predicted probability of having at least one therapeutic LP during follow-up divided by the conditional predicted probability of having at least one therapeutic LP, conditional on possible confounders. Stabilized weights for censoring were estimated in a similar fashion considering the same set of factors for the conditional probability. The final stabilized weights (sw_i) were the product of the stabilized exposure weights and the stabilized censoring weights [74]. The mean of the sw_i should be centered on 1.0 with low standard deviation.

Confounders were defined as baseline factors that were associated with exposure and also related to 11-day mortality or factors that changed the estimated relative risk by

more than 10% when adjusted for. Three-knot splines, categorical transformations, and linear forms of the confounders were considered when constructing the inverse probability weights [75]. The final form for confounders was chosen considering the trade-off between flexibility to account for residual confounding and reduction in model standard error. From this process, linear terms for the intercept and continuous covariates were thought to allow the most flexibility without increasing standard errors. Glasgow Coma Scores were dichotomized as <15 or 15 for all models.

P-values <0.05 were considered statistically significant. All data processing and analyses were conducted in SAS version 9.3 (SAS Institute, Cary, NC).

3.2.4 Missing Data

Because of the occurrence of missing baseline data, multiple imputation was conducted to better estimate the effect of therapeutic LPs on acute mortality. The most common missing factor was weight (missing for 34% of subjects), due to the inability of many of the individuals to stand on their own for effective measurement. Other common missing factors were CSF opening pressure (16% missing), serum potassium (12% missing), and complete blood counts (8% missing). All other variables were missing in less than 8% of the sample. Reasons for missing data stemmed from insufficient supplies or sample volumes to get valid measures. As such, missing data were assumed to be missing at random; that is, the likelihood of a parameter being missing was not thought to be dependent on the unobserved value of the parameter [76] . One exception may be missing weight, as the inability to stand for adequate weight measurement is possibly related to severity of illness and short-term mortality. Sensitivity analyses considering models with an indicator variable for missing weight and with weight excluded from the models were explored.

A Markov Chain Monte-Carlo process was used for multiple imputation of missing parameters and 40 full datasets were imputed. The model used for imputation included

baseline characteristics of age, sex, CSF opening pressure, CSF closing pressure, CSF cryptococcal quantitative culture, CSF white blood cells (WBC), amount of CSF removed during diagnostic LP, CSF protein, hemoglobin, peripheral WBC, red blood cells, platelets, serum creatinine, hematocrit, potassium, mean corporal volume, headache, duration of headache prior to admission, neck pain, duration of neck pain prior to admission, occurrence of seizures, decreased consciousness, nausea, photophobia, papilledema, wasting, Karnofsky score, systolic and diastolic blood pressure, heart rate, respiratory rate, temperature, pulse oximetry, weight, Glasgow Coma Score, exposure to therapeutic LPs, and death within 11 days of CM diagnosis. All data with missing values received an imputed value. Exposure to at least one therapeutic LP during follow-up and vital status were included to improve the prediction of missing parameters; however, no individuals was missing exposure or vital status and no imputation was done for these parameters.

3.3 Results

3.3.1 Study Population

A total of 248 individuals were included in this analysis and were observed for a total of 1698 person-days (figure 3.1). Subjects with cryptococcal meningitis (CM) in this cohort were, on average, 36 years old, 55% were male, 29% had altered mental status (Glasgow Coma Scale (GCS) <15), and had had a headache for a median of 2 weeks before admission.

Seventy-five (30%) subjects received at least one therapeutic LP after the diagnostic LP (table 3.1). Subjects who had at least one therapeutic LP weighed slightly more, had no observed papilledema, lower heart rate, were less likely to be febrile, had slightly lower creatinine, and were slightly more likely to be missing weight at CM diagnosis. Additionally, those with at least one therapeutic LP had higher CSF fungal burden, higher CSF opening and closing pressure, and more CSF was removed during the first diagnostic

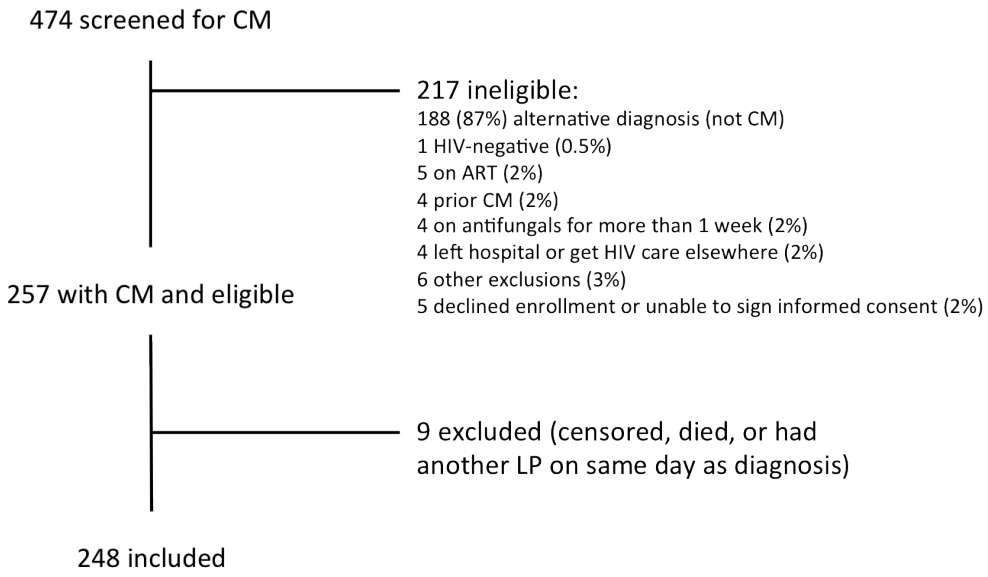


Figure 3.1: Selection of cohort participants among HIV-positive, ART-naïve individuals in South Africa and Uganda screened for cryptococcal meningitis.

Table 3.1: Baseline characteristics and mortality by therapeutic lumbar punctures among HIV-positive, ART-naïve individuals with cryptococcal meningitis in South Africa and Uganda.

		At least one therapeutic LP		**No therapeutic LP**		
		N with data	**Median (IQR) or N (%)** [a]	N with data	**Median (IQR) or N (%)** [a]	**P-value** [b]
N per group			75 (30%)		173 (70%)	
Site [c]		75		173		0.02
	Kampala		61 (34%)		120 (66%)	
	Mbarara		4 (11%)		34 (89%)	
	Cape Town		10 (35%)		19 (65%)	
Age (years)		75	34 [29, 40]	173	37 [30, 42]	0.15
Males, N (%)		75	44 (59%)	173	91 (53%)	0.38

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Table 3.1 – continued from previous page

	At least one therapeutic LP		No therapeutic LP		
	N with data	**Median (IQR) or N (%)** [a]	N with data	**Median (IQR) or N (%)** [a]	**P-value** [b]
Weight (kg)	43	57 [46, 62]	121	52 [45, 57]	0.08
Missing weight	75	32 (43%)	173	52 (30%)	0.05
Headache duration	72		169		0.56
less than 7 days		8 (11%)		18 (11%)	
7-13 days		27 (38%)		50 (30%)	
14-20 days		16 (22%)		35 (21%)	
21-27 days		5 (7%)		22 (13%)	
28 days or more		16 (22%)		44 (26%)	
Papilledema, N (%)	71	0 (0%)	163	8 (5%)	0.06
Karnofsky Score	74	50 [40, 50]	173	50 [40, 60]	0.16
Glasgow Coma Scale, N (%)	74		173		0.15
< 15		26 (35%)		45 (26%)	
15		48 (65%)		128 (74%)	
Heart rate (beats per minute)	74	76 [66, 90]	172	81 [72, 97]	0.01
Respiratory rate (breaths per minute)	71	20 [20, 24]	172	22 [20, 24]	0.40
Systolic blood pressure (mmHg)	74	114 [109, 122]	169	110 [103, 122]	0.14
Diastolic blood pressure (mmHg)	74	70 [60, 80]	169	70 [60, 82]	0.70
Axillary temperature (^{o}C)	74	36.4 [36.0, 36.9]	171	36.5 [35.9, 37.5]	0.30
Fever (axillary temperature $>$ 37.5^o C)	74	9 (12%)	171	40 (23%)	0.04
Clinical Laboratory Values					
Hemoglobin (g/dL)	71	11.5 [9.4, 13.0]	155	11.0 [8.9, 13.0]	0.41
Hematocrit (%)	71	32.8 [29.0, 38.2]	155	33.2 [27.2, 38.6]	0.75
White blood cells (x$10^3/\mu$L)	71	3.7 [2.6, 5.3]	155	3.4 [2.5, 5.1]	0.41
Creatinine (mg/dL)	73	0.6 [0.5, 0.8]	160	0.7 [0.6, 0.9]	0.03

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Table 3.1 – continued from previous page

	At least one therapeutic LP		No therapeutic LP		
	N with data	**Median (IQR) or N (%)** [a]	N with data	**Median (IQR) or N (%)** [a]	**P-value** [b]
Potassium (mmol/L)	63	3.9 [3.5, 4.3]	156	4.0 [3.5, 4.3]	0.83
CSF Parameters					
Opening pressure (mmH_2O)	69	346 [220, 440]	139	248 [150, 338]	<0.001
Opening pressure >250 mmH_2O, N (%)	69	48 (70%)	139	69 (50%)	0.007
Closing pressure (mmH_2O)	64	100 [80, 137]	126	90 [60, 120]	0.04
Amount of CSF removed during lumbar puncture (mL)	72	19 [12, 27]	168	14 [8, 20]	<0.001
Quantitative cryptococcal culture ($\log_{10}$ CFU/mL)	75	5.3 [4.4, 5.6]	159	5.0 [3.9, 5.5]	0.03
White blood cells ($/\mu$L)	74	10 [<5, 105]	158	10 [<5, 85]	0.73
White blood cells < 5 cells/μL	74	34 (46%)	158	66 (42%)	0.55
Number of lumbar punctures, N (%)	75		173		
None		–		173 (100%)	
1		60 (80%)		–	
2 or more		15 (20%)		–	
Outcome					
Died, N (%)	75	5 (7%)	173	31 (18%)	

[a] Median and interquartile range (IQR, 25^{th} - 75^{th} percentile). Percentages are column percentages, unless otherwise noted.

[b] P-values from chi-square test for frequencies and Wilcoxon ranksum test for medians.

[b] Row percentages are presented.

LP compared to those who did not have a subsequent LP. All other clinical and demographic parameters were similar between the two groups.

The majority of subjects (80%) who had at least one therapeutic LP underwent only

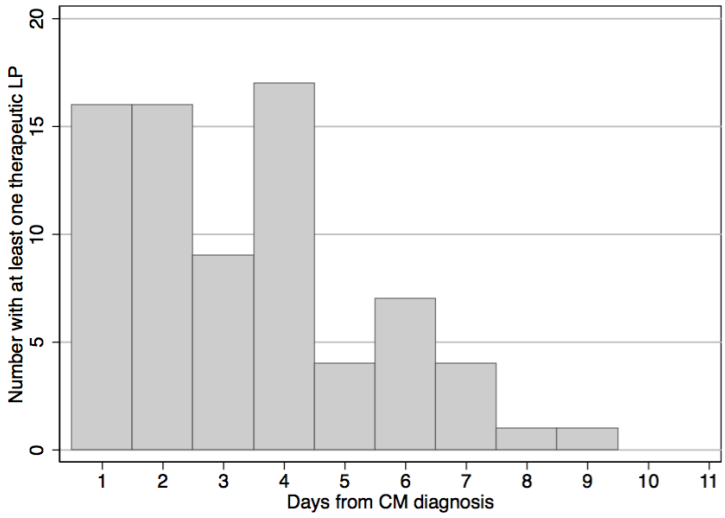


Figure 3.2: Distribution of days from diagnosis of cryptococcal meningitis to the first therapeutic LP among HIV-positive, ART-naïve patients in South Africa and Uganda.

one LP during follow-up. The median time to first therapeutic LP was 3 days after CM diagnosis (25^{th} to 75^{th} percentile: 2 to 4 days, figure 3.2). The occurrence of therapeutic LPs was slightly differential by study site with individuals in Kampala and Cape Town tending to be more likely to get a therapeutic LP. Of note among those without an additional LP during follow-up, 50% had CSF opening pressures of at least 250 mmH_2O at CM diagnosis.

The median CSF opening pressure measured at the first therapeutic LP was 270 mmH_2O (25^{th} to 75^{th} percentile: 180 to 401, among 70 subjects with measurements) and 40 individuals (57%) had opening pressures greater than 250 mmH_2O at the first therapeutic LP, suggesting that the LP was needed to reduce the pressure in over half of the individuals. Fifteen individuals (20% of all with an LP and 6% of all subjects) had at least 2 therapeutic LPs, 2 individuals had 7 LPs, and 1 individual had 8 LPs within the first 11 days of CM diagnosis.

3.3.2 Acute Mortality

Thirty-six deaths were observed within 11 days of CM diagnosis (15%) for an overall mortality rate of 2.1 per 100 person-days (95% CI: 1.5 - 2.9 per 100 person-days). The median time to death was 4 days (25^{th} to 75^{th} percentile: 2 to 6 days). Acute mortality was associated with lower weight (median 43 kg among those who died versus 54 kg among those censored, p-value = 0.007), missing weight at baseline (58% versus 30%, p-value <0.001), lower GCS (44% versus 26% with GCS <15, p-value = 0.03), greater heart rate (92 versus 80 beats per minute, p-value = 0.003), faster respiratory rate (24 versus 20 breaths per minute, p-value = 0.003), and higher CSF fungal burden (median 5.3 versus 5.1 $\log_{10}$ CFU/mL, p-value = 0.04) at CM diagnosis.

The CSF opening pressure at the time of CM diagnosis did not differ for those who died versus those censored (respectively, median CSF OP 290 versus 265 mmH_2O, p-value = 0.92; 59% and 56% with pressures $\geq$250 mmH_2O, p-value = 0.74) and the amount of CSF removed during the first diagnostic LP was similar between those who died and those who were censored (respectively, median CSF removed 18 mL versus 15 mL, p-value = 0.47). Also unassociated with mortality was the presence of CSF inflammation, as measured by CSF WBC; 55% of those who died had undetectable CSF WBC (< 5 /μL) compared to 41% among those censored (p-value = 0.15). Mortality rates were slightly higher in Kampala (17% or 2.5 per 100 person-days, 95% CI: 1.8 - 3.6 per 100 person-days), but were not significantly higher than in Mbarara (8% or 0.3 per 100 person-days, 95% CI: 0.07 - 0.7 per 100 person-days) or Cape Town (7% or 0.2 per 100 person-days, 95% CI: 0.04 - 0.7 per 100 person-days; p-value = 0.16).

Of those who did not have a therapeutic LP, 18% (31 of 173) died within 11 days of CM diagnosis for a mortality rate of 2.4 per 100 person-days (95% CI: 1.6 - 3.3 per 100 person-days), whereas 7% (5 of 75) who had at least one therapeutic LP died for a

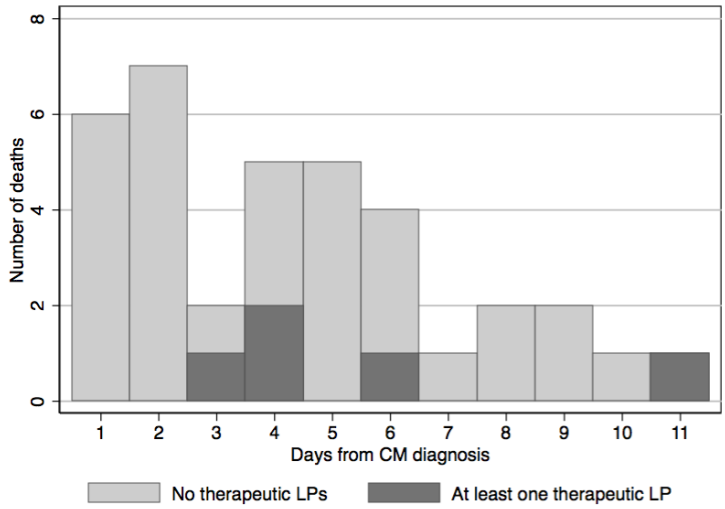


Figure 3.3: The distribution of days from diagnosis of cryptococcal meningitis to death among those with and without at least one therapeutic LP among HIV-positive, ART-naïve individuals in South Africa and Uganda

mortality rate of 1.3 per 100 person-days (95% CI: 0.5 - 2.9 per 100 person-days; figure 3.3). All individuals receiving at least 2 therapeutic LPs were censored at a median of 7 days (6 to 11 days). Of those who died, all 5 individuals with a therapeutic LP underwent only 1 additional LP during the first week following CM diagnosis.

3.3.3 Multivariable Association

In a crude, unweighted pooled regression, which accounts for the time-varying nature of therapeutic LPs, the relative risk (RR) of mortality was 0.5 with at least one compared to no therapeutic LP (95% CI: 0.2 - 1.4; table 3.2). Stepwise addition of heart rate, CSF fungal burden, and an indicator for low GCS resulted in more extreme relative risks (adjusted models 1-3) and adjustment for CSF closing pressure and weight had no additional impact on the relative risk estimate (adjusted model 4 and fully adjusted model). Adjustment for

Table 3.2: Estimated relative risk of acute mortality among those receiving a therapeutic lumbar puncture within 11 days of diagnosis of cryptococcal meningitis in HIV-positive, ART-naïve patients in South Africa and Uganda.

	Relative Risk	95% CI
Crude model	0.53	(0.20, 1.37)
Marginal Structural Model Pooled Poisson Regression [a]		
Adjusted model 1	0.50	(0.19, 1.32)
Adjusted model 2	0.39	(0.14, 1.07)
Adjusted model 3	0.31	(0.12, 0.82)
Adjusted model 4	0.31	(0.11, 0.88)
Fully adjusted model [b]	0.33	(0.11, 1.03)
Unweighted Pooled Poisson Regression		
Adjusted model 1	0.64	(0.24, 1.70)
Adjusted model 2	0.52	(0.19, 1.38)
Adjusted model 3	0.47	(0.17, 1.26)
Adjusted model 4	0.47	(0.17, 1.28)
Fully adjusted model [b]	0.50	(0.18, 1.36)

Confidence interval (CI); Glasgow Coma Score (GCS).
[a] Adjusted model 1 adjusted for heart rate.
Adjusted model 2 is adjusted model 1 additionally adjusted for CSF fungal burden.
Adjusted model 3 is adjusted model 2 additionally adjusted with an indicator for GCS <15.
Adjusted model 4 is adjusted model 3 additionally adjusted for CSF closing pressure.
[b] Fully adjusted model accounts for heart rate, CSF fungal burden, indicator for GCS <15, CSF closing pressure, and weight.

CSF opening pressure also did not result in measureable changes in the relative risk (data not shown).

Excluding extraneous covariates and adjusting for heart rate, CSF fungal burden, and GCS, adjusted model 3 appeared to be the best model choice. From this model, it is estimated that the average effect of therapeutic LP was to reduce the risk of mortality by 69% (RR = 0.3, 95% CI: 0.1 - 0.8). The mean stabilized weights (sw_i) for adjusted model 3 was 1.02 (standard deviation (SD) = 0.52). The unweighted relative risk estimate for adjusted model 3 was slightly attenuated from the marginal structural model estimate, and did not reach statistical significance.

3.3.4 Opening Pressure Subgroups

Exploratory subgroup analysis was conducted to assess whether the effect of therapeutic LPs differed by the baseline CSF opening pressure. One hundred seventeen individuals had high baseline opening pressure (56% of those with baseline opening pressures) and 14% of this subgroup died within 11 days of CM diagnosis; whereas, 91 individuals had opening pressures less than 250 mmH_2O (44% of those with pressures) and 12% of this subgroup died within 11 days of CM diagnosis. The frequency of therapeutic LPs appeared to be higher in the subgroup with high baseline pressures than in the subgroup with lower pressures (table 3.3). No deaths were observed among 21 individuals with baseline pressures less than

Table 3.3: Association of therapeutic lumbar puncture and acute mortality in HIV-positive, ART-naïve patients with cryptococcal meningitis by baseline CSF opening pressure.

	At least one therapeutic LP	No therapeutic LP	Overall
Baseline CSF Opening Pressure < 250 mmH_2O			
Number of individuals (% of overall)	21 (23%)	70 (77%)	91
Deaths, N(%)	0 (0%)	11 (16%)	11 (12%)
Person-days of observation	98	554	652
Mortality rate (per 100 person-days)	0	1.99	1.69
Unadjusted relative risk (95% CI) [a]	0.00 [0.00, 2.25]		
Baseline CSF Opening Pressure ≥ 250 mmH_2O			
Number of individuals (% of overall)	48 (41%)	69 (59%)	117
Deaths, N(%)	4 (8%)	12 (17%)	16 (14%)
Person-days of observation	260	505	765
Mortality rate (per 100 person-days)	1.54	2.38	2.09
Unadjusted relative risk (95% CI)	0.65 [0.15, 2.14]		
Baseline CSF Opening Pressure Not Available			
Number of individuals (% of overall)	6 (15%)	34 (85%)	40
Deaths, N(%)	1 (17%)	8 (24%)	9 (23%)
Person-days of observation	28	253	281
Mortality rate (per 100 person-days)	3.57	3.16	3.20
Unadjusted relative risk (95% CI)	1.13 [0.03, 8.42]		

Lumbar puncture (LP); Confidence interval (CI).
[a] A value of 0.5 was added to both the numerator and denominator for the group with at least one therapeutic LP in order to estimate the rate and confidence intervals.

250 mmH_2O who received at least one therapeutic LP. Though this subgroup analysis was largely exploratory, the stratified relative risks were not vastly different from the relative risk in the entire cohort providing little evidence to suggest that the effect of therapeutic LPs was only restricted to individuals with higher baseline opening pressure, as may be anticipated.

Forty individuals did not have CSF opening pressure measured at the time of CM diagnosis. The main reason patient records were missing this variable was because the initial lumbar puncture was conducted by non-study staff who either did not use a manometer to measure the pressure or failed to record the value. Baseline characteristics were similar among those who did and did not have opening pressure measured, aside from a greater amount of CSF removed during the diagnostic LP in those for whom opening pressure was measured (median 8mL removed in those without opening pressure versus 16 mL in those with measurements, p-value < 0.001; see Appendix A, section A.2). Of the 40 individuals with missing opening pressure, far fewer received therapeutic LPs during follow-up, only 15% compared to 23% with lower pressure and 41% with high pressure. Overall, the mortality seemed higher among those without pressure measurements compared to individuals with measured pressures and there seemed to be higher mortality particularly among those who received at least one therapeutic LP; however, this subgroup was too small to provide conclusive evidence for or against therapeutic LPs in those without measured CSF opening pressure.

3.4 Conclusions and Discussion

Raised intracranial pressure (ICP) is common in cryptococcal meningitis (CM), occurring in over 60% of patients in sub-Saharan Africa [30, 50]. Raised ICP is thought to be due to blockage of CSF drainage in the arachnoid villi and granulations by masses of cryptococcal

cells, inflammation, or a combination of these factors [15, 16, 51, 55]. Several studies have commented on the possible link between raised ICP and short-term mortality in CM [15, 50, 51, 68].

Early during the HIV epidemic in the US, a CM treatment study found that 58% of 221 patients with measured CSF opening pressure had pressures greater than 250 mmH_2O [51]. The authors described greater clinical failure rates, but similar mycologic failure rates, at the end of therapy among patients who experienced increases in CSF opening pressure than among those who had no change or a decrease in pressure over two weeks of therapy. In Kaplan-Meier analysis, the authors found that those with baseline CSF opening pressures of at least 250 mmH_2O had shorter time to death and lower 12-month survival was observed with increasing CSF opening pressure quartiles. The derived relative risk of 2-week mortality in the US-cohort was 9.4 comparing the group with baseline opening pressures $\geq$250 mmH_2O to those with pressures $<$ 250 mmH_2O (95% CI: 1.2 - 71.8).

In a Ugandan cohort of 126 CM patients with baseline CSF opening pressure measurements, those with baseline opening pressures of at least 250 mmH_2O had 2.1 times greater odds of 2-week mortality than those with lower CSF pressures (95% CI: 0.9 - 5.2) [30]. Similarly, a cohort of 161 CM patients in Thailand and South Africa found that, among the 137 with baseline opening pressure measurements, the relative risk of 2-week mortality was 1.5 times greater for those with baseline opening pressures $\geq$200mmH_2O (95% CI: 0.6 - 4.1) [50]. These studies may have been underpowered to detect the effect of pressure on mortality, as evidenced by the large confidence intervals and neither of these studies quantified the association between use of repeated lumbar punctures potentially preventing these deaths.

With the evidence that higher pressures are associated with greater risk of mortality, the American [37], and now the international [38], treatment guidelines for cryptococcal meningitis recommend measuring CSF opening pressure in all patients with CM and closely

monitoring for changes in pressures. Interventions to reduce pressures should include recurrent, possibly daily, lumbar punctures when pressures are greater than 250 mmH_2O. The cohorts from Thailand and South Africa, as well as a commentary from field experiences, attribute reductions in mortality to aggressive control of CSF pressures through repeat lumbar punctures and lumbar drains [50, 69]; though no studies have directly assessed the effect of interventions to reduce ICP on CM mortality. One pilot trial, conducted in Uganda, found that daily LPs compared to LPs at diagnosis, day 7, and day 14 of CM treatment led to declines in CSF opening pressure (mean change of 63 mmH_2O in the daily LP group versus 46 mmH_2O change in the periodic LP group) but was underpowered to assess differences in mortality [70].

The objective of this analysis was to quantify the average effect of intervening to reduce intracranial pressure (ICP) on acute mortality after diagnosis of CM. To achieve this objective, HIV-positive, ART-naïve individuals diagnosed with a first episode of CM were prospectively observed, during the initial stages of in-hospital treatment, for the frequency of therapeutic LPs and mortality. Among the 248 participants included in this cohort, 30% received at least one therapeutic LP and 15% died within 11 days. The majority of those who died (85%) did not have an LP during observation after the initial LP leading to CM diagnosis. Once the time-varying nature of the exposure and possible confounders were accounted for, the estimated average effect of therapeutic LPs was to lower mortality by 69% (95% CI: 18% - 88%).

A marginal structural modeling approach, accounting for time-varying exposure, was used to estimate the average effect of therapeutic LPs. This modeling approach adjusts for confounding through weighting by the inverse joint probability of exposure and censoring. Doing so creates a pseudo-population in which there is no association between the exposure, or censoring, and covariates used to construct the weights. The main effect of exposure is then estimated using the pseudo-population, where no confounding by the

included covariates is present. The result of this method is an estimate of the total effect of therapeutic LPs, in a population with the observed distribution of clinical and demographic factors, had all individuals received at least one therapeutic LP versus when no one received therapeutic LPs. Conversely, unweighted, multivariable-adjusted regression, the conventional approach in observational studies, yields the estimated effect of an LP in a population where everyone gets at least one LP compared to when no one gets an LP and where all covariate levels are held constant in the population.

The major difference between the conventional approach and the marginal structural model is whether the estimated effect is assumed to be constant across baseline covariates (as in conventional regression) or averaged over levels of covariates (as in marginal structuals). The added benefit of estimating the effect averaged over level of baseline covariates is that the point estimate more realistically reflects the effect of an intervention applied to the target population as a whole, as opposed to assuming constancy of the effect within strata of covariates. Both analytic methods, in this analysis, yielded similar point estimates between 36 to 69% reductions in mortality risk with at least one therapeutic LP. However, the marginal structural approach resulted in a model demonstrating statistical signficance, whereas the conventional adjusted regression indicated non-significance.

The results of this analysis suggest there is a benefit to undergoing at least one therapeutic LP to help control ICP among all CM patients. Prior data suggests that ICP can build up over time and may be raised without symptoms [50, 51]. Therefore, the observed survival benefit may be due to further reducing the ICP before it has built up to the point of causing overt symptoms and physiologic damage. There was no evidence in this cohort that the effect of therapeutic LPs was relegated to those with high baseline opening pressure, further suggesting that all CM patients may benefit from additional intervention to control ICP. It was also notable that no deaths were observed after receiving a therapeutic LP among individuals with normal baseline opening pressures. As the current subgroup

analysis by baseline opening pressure is small and likely underpowered, future investigation is needed to understand whether the benefits of relieving ICP during CM treatment are broadly experienced and whether additional LPs should be considered for all CM patients not just patients with high baseline pressures or those who develop symptoms.

Few individuals received more than one therapeutic LP during the observation period, so it was not possible to assess the effect of more than one therapeutic LP on acute mortality; however, no deaths were observed in the subset of individuals who did receive more than one therapeutic LP in the first 11 days after CM diagnosis. While anecdotally this alludes to possible benefits of additional LPs in the first week of CM therapy, clearly more investigation is needed to understand whether more LPs would be beneficial for all patients.

A limitation to this analysis is the potential for unmeasured confounding. More formally, there is the possibility that factors causally related to mortality and differentially distributed between the therapeutic LP groups were unmeasured and therefore uncontrolled. This limits the causal interpretation and internal validity of the point estimate. The current analysis attempted to adjust for all potential confounders, including the baseline mental status that has been linked with 2-week mortality in CM patients in numerous cohorts [30, 51, 64, 77, 78], and additional indications for a lumbar puncture. However, data on the clinical status of individuals were collected only at baseline and, though patients were seen daily by the attending study clinicians, these data were not updated daily. Characteristics such as loss of visual or auditory acuity, worsening headache, or declines in mental status over the observation period may have occurred more frequently among individuals who died quickly. If these characteristics were also more common among those who did not receive an LP – perhaps because the patient declined an LP or the clinician decided against an LP because of the perceived risk to the patient or perceived lack of benefit for moribund individuals – then uncontrolled confounding may be present. This source of bias

cannot be excluded because no data were available on whether an LP had been considered for an individual. Although it is unknown whether such confounding factors exist, it is reasonable to assume that LPs were being uniformly considered for all subjects, because the same attending clinician was seeing patients within a site. Uniformity in practice might reduce the potential for an association to exist between symptoms of increased ICP, such as worsening headache and declining mental status, and mortality.

One approach to assess for residual confouding was to evaluate the estimated effect of therapeutic LPs stratified by baseline opening pressure, perhaps the greatest indicator for undergoing additional LPs. As described above, both the group with high baseline pressure as well as those with normal pressures exhibited lower mortality in the group that underwent at least one therapeutic LP. Although this assessment does not formally negate the potential for confounding, and is limited in power, it is reassuring that one subgroup did not experience a widely different effect from the other strata or from the overall cohort.

Further investigation of the use of therapeutic LPs among CM patients is warranted to see if a possible beneficial effect is uniformly experienced in different settings and among all patients without any adverse effects. Relatedly, future studies should examine the impact of syndromic versus systematic intervention on ICP, such as preliminarily investigated by Orem, et al. [70]. For example, patients in the current study were considered for additional LPs based on baseline CSF opening pressure and the existence of symptoms of raised ICP over the course of treatment. Other studies, on the other hand, have taken a more systematic approach and conducted therapeutic LPs at day 3, 7, and 14 after CM diagnosis regardless of baseline pressure and symtoms over treatment. Such studies have failed to find associations between baseline CSF opening pressure and mortality [50], and it is possible that the lack of association is because of the survival benefit from therapeutic LPs regardless of CSF opening pressure described by this analysis. In conclusion, the findings from this analysis support the current American and WHO guidelines for lumbar punctures to help

diagnose CM as well as to reduce ICP and suggest reduced risk of death when patients receive at least one therapeutic lumbar puncture during the first week of CM treatment.

Chapter 4

Predictive Factors of CSF sterility at the End of Amphotercin-based Therapy for Cryptococcal Meningitis

Introduction Amphotericin B is an effective antifungal drug to treat cryptococcal meningitis (CM), however it has numerous toxic side effects. To avoid some of these toxic effects, recent studies have advocated for reduced duration of amphotericin treatment for CM. A potential disadvantage to reducing treatment duration in all patients is that many will be left with residual infection, which has been linked with detrimental sequelae including greater rates of CM relapse and immune reconstitution inflammatory syndrome. The objective of this analysis was to describe clinical and demographic factors related to a sterile cerebrospinal fluid (CSF) culture after two weeks of amphotericin-based antifungal therapy with the aim of describing profiles of patients who may be candidates for shorter

amphotericin regimens.

Methods HIV-positive, antiretroviral therapy (ART)-naïve patients with a first episode of CM enrolled in the Cryptococcal Optimal ART Timing (COAT) trial were included in this analysis. CM was diagnosed by CSF culture or CSF cryptococcal antigen (CRAG) detection. All patients were treated with two weeks of daily amphotercin and 800mg fluconazole, followed by stepped-down doses of daily fluconazole. Clinical and demographics factors were assessed at the time of CM diagnosis and compared between individuals who achieved a sterile CSF culture by the end of amphotericin therapy and individuals who did not achieve a sterile culture, using binary logistic regression. Cut-points of baseline CSF quantitative culture burden (in colony-forming units (CFU)/mL) and baseline CSF CRAG titers were explored for their ability to predict CSF sterility. Optimal cut-points were chosen based on maximizing specificity and considering increased sensitivity.

Results One hundred seventy-seven CM patients were enrolled in COAT, of whom 13 died before sterility could be observed, 23 did not have a final culture to assess sterility, 57 had a final culture that was not sterile, and 81 had a sterile culture by the end of two weeks of amphotericin-based therapy. Those with a sterile culture were more likely to have lower fungal burden by culture or CRAG titer at baseline, lower baseline hemoglobin, and a faster rate of cryptococcal clearance during the first week of therapy compared to individuals who did not have a final sterile culture. Cut-points of baseline fungal burdens $\leq$10,000 CFU/mL or $\leq$1:1024 CSF CRAG titer were good indicators, based on sensitivity and specficity, of the probability of CSF sterility at the end of amphotericin induction therapy.

Conclusions Inidividuals with lower cryptococcal burdens at CM diagnosis had significantly higher odds of having a sterile CSF culture by the end of amphotericin-based

antifungal therapy. This evidence suggested that therapy could be customized on the basis of baseline fungal burden, and those with lower burdens of infection may not need a full two weeks of amphotericin therapy, as sterility was observed in the majority of these patients prior to the end of therapy. However, two weeks of potent induction therapy may be needed for the average patient in sub-Saharan Africa.

4.1 Introduction

Effective antifungal treatment has vastly reduced mortality from HIV-associated cryptococcal meningitis (CM). The workhorse of antifungal therapy for CM is amphotericin B and current treatment guidelines suggest amphotericin-based treatment for two weeks after diagnosis [37, 38]. Such treatment strategies result in roughly 80-90% survival after two weeks in HIV-positive individuals [29–31].

Amphotericin B is an effective antifungal drug, however has numerous toxic side effects, including nephrotoxicity, acute renal failure, and electrolyte abnormalities [79–81]. To avoid toxicity, recent studies have evaluated reduced duration of amphotericin treatment for CM [57,59,82]. Reducing the amphotericin duration to 5 to 7 days, in conjunction with high dose fluconazole (1200 mg/day) for the initial, induction phase of therapy, seemed to confer similar rates of clearance of *Cryptococcus neoformans* from the CSF, in phase II trials compared to a 14-day regimen, while limiting toxicities [57, 59] and reducing costs [82].

One disadvantage to treating CM patients with a shortened course of amphotericin is that a large proportion of individuals will not achieve CSF sterility by the end of the amphotericin treatment. Despite two weeks of amphotericin-based treatment, 50-70% of HIV-positive individuals will continue to have viable *C. neoformans* detectable in their CSF at the end of treatment [25, 40, 41]. Residual infection may result in increased incidence of long-term sequelae, including persistent symptoms [25, 41], symptomatic relapse [83],

HIV-associated immune reconstitution inflammatory syndrome [43,58], and possibly higher long-term mortality [43]. An open question remains whether short-course amphotericin is broadly applicable or should be used only in patients with a good prognosis of achieving a sterile CSF before the end of the traditional 2-week treatment phase. Therefore, the focus of this paper is to further understand factors predictive of successful CSF sterility and assess whether it might be feasibile to tailor therapy based on the most predictive factors.

Reasons for not achieving CSF sterility by the end of amphotericin treatment have been described in the US, France, and South Africa and are related to treatment with less effective amphotericin dosing, high baseline fungal burdens, disseminated infection (i.e. *C. neoformans* found on blood culture), lower serum creatinine, low serum albumin levels, absence of fever, and high serum and CSF titers of cryptococcal antigen [25, 40, 41, 43]. These studies provide a foundation for understanding factors related to failure to reach CSF sterility, namely, that the severity of cryptococcal infection and an insufficient immune response, as indicated by lack of fever and high levels of *C. neoformans*, may contribute to the probability that amphotericin therapy will not completely sterilize the CSF in two weeks. However, prior studies were conducted several years ago, and, currently, only one study has described the clinical factors predictive of CSF sterility after CM treatment in sub-Saharan Africa [43]. The current analysis adds to the existing literature to augment the understanding of factors predictive of CSF sterility at the end of amphotericin treatment for HIV-associated CM in African populations.

4.2 Methods

4.2.1 Study Population and CM Treatment

Subjects included in this analysis were enrolled in the Cryptococcal Optimal Antiretroviral (ART) Timing (COAT) trial, a randomized clinical strategy trial for HIV-positive, ART-naïve persons with a first episode of CM to determine whether early ART initiation (1-2 weeks after CM diagnosis; prior to hospital discharge) results in superior 26-week survival compared to deferred ART initiation (5 weeks after diagnosis; as an outpatient). Individuals were eligible for enrollment and randomization in the COAT trial if they were at least 18 years old, receiving amphotericin-based CM treatment, willing to attend regular clinic visits, and provided informed consent. Individuals who were pregnant, breastfeeding, on immunosuppressive therapy, unable to take oral medication, had contraindications to study medications, had been on antifungal therapy for more than one week, or had a prior episode of CM were all excluded. Screening began in November 2010 and ended in April 2012, during which time 177 individuals were randomized from three sites: Mulago Hospital/Infectious Diseases Institute in Kampala, Uganda; Mulago-Mbarara Joint AIDS Program and Mbarara University of Science and Technology in Mbarara, Uganda; and GF Jooste Hospital in Cape Town, South Africa. Approval for the COAT trial was granted by the research ethic committees at the University of Minnesota, Makerere University, Mulago Hospital, Mbarara University, University of Cape Town, Uganda National Council of Science and Technology, South African Medicines Control Council, and National Institutes of Health's National Institute for Allergy and Infectious Diseases Clinical Science Review Committee (Clinicaltrials.gov: NCT01075152). Approval for this analysis was additionally granted by the Institutional Review Board at the University of Minnesota.

Diagnosis of CM was made after a positive CSF cryptococcal culture or CSF cryptococcal antigen (CRAG) latex agglutination assay. Induction treatment consisted of daily

0.7-1.0 mg/kg amphotericin B in combination with 800 mg fluconazole for two weeks. All individuals also received intravenous fluids and electrolyte supplementation during amphotericin therapy. Therapeutic lumbar punctures to control intracranial pressure were conducted according to treatment guidelines [37]; in addition, the COAT protocol specified additional lumbar punctures to be done after 7 and 14 days of amphotericin to monitor clearance of *C. neoformans*. Regardless of sterility at the end of amphotericin therapy, all individuals received 3 further weeks of 800 mg/day fluconazole followed by 8 weeks of 400 mg/day fluconazole and at least one year of 200 mg/day fluconazole.

Randomization of eligible individuals occurred after 7 to 11 days of amphotericin therapy. Those randomized to the early arm initiated ART therapy within 48 hours of randomization, while those randomized to the deferred arm initiated ART 4 weeks after randomization. All individuals initiated combination ART with either efavirenz plus zidovudine and lamivudine or efavirenz plus stavudine and lamivudine.

4.2.2 CSF Quantitative Cultures and Definitions of CSF Sterility

All CSF samples obtained from lumbar punctures during amphotericin therapy, including the LP conducted at time of CM diagnosis, were evaluated by qualitative and quantitative fungal culture. Cultures were conducted in microbiology labs, approved by the US National Institutes of Health, at each study site. Briefly, CSF was plated onto Sabouraud's dextrose agar and incubated at 30^{o}C to allow fungal growth. Cultures were qualitatively considered positive when *C. neoformans* colonies were detected or negative in the absence of fungal growth after 14 days of incubation. Quantification of *C. neoformans* fungal burden was conducted using 10-fold serial dilutions of CSF, up to $1{:}10^5$ dilution, and counting colony-forming units (CFUs) of *C. neoformans* on the most dilute plate with growth [29]. Quantitative colony-forming units/mL were $\log_{10}$ transformed for analysis.

Inidividuals were categorized into three mutually exclusive groups; sterile, non-sterile,

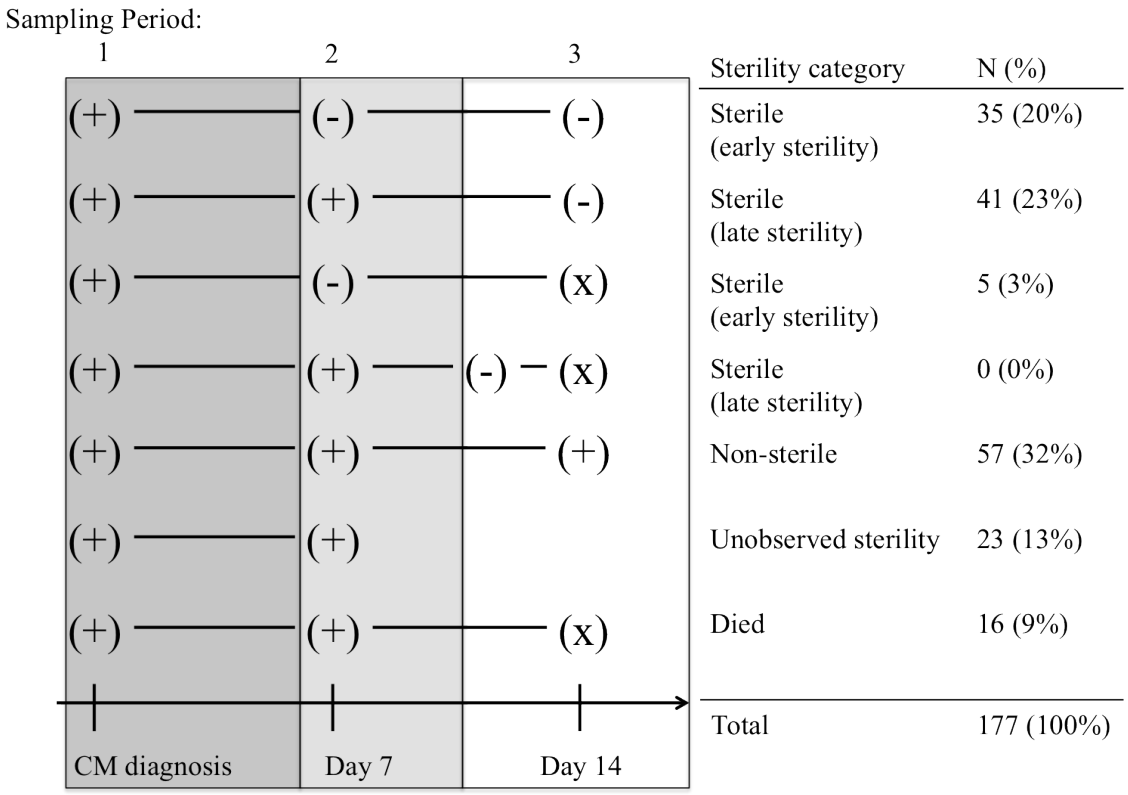


Figure 4.1: Sterility outcomes for individuals with cryptococcal meningitis in the COAT trial. The left side of the figure depicts the results of CSF cultures and vital status((+) = alive with positive culture, (-) = alive with negative culture, and (x) = dead) during sampling periods. The first sampling period extended from CM diagnosis until day 7 of amphotericin-based therapy, the second period extended from day 7 to day 11 of therapy, and the third period extended from day 12 until the end of amphotericin-based therapy.

and died (figure 4.1). The sterility category was determined based on all observed culture results during induction therapy. For simplicity, three sampling periods were defined. The first sampling period extended from the LP done at CM diagnosis until day 7 of amphotericin, the second period extended from day 7 to 11, and the third period extended from day 12 to the end of amphotericin therapy. Individuals were classified as having reached CSF sterility when a CSF culture was negative and no subsequent positive cultures were found for the remainder of amphotericin induction therapy. Ten individuals were found to

have a negative culture followed by a positive culture later during amphotericin therapy. The initial negative cultures for these individuals were considered to be false negatives. Such culture reversions accounted for 0.7% of all observed cultures. Individuals found to have a sterile culture during amphotericin treatment but who died before treatment ended were considered to have reached the CSF sterility endpoint.

Non-sterility was defined for individuals in whom no negative cultures were observed during amphotericin therapy but for whom a culture was observed between day 12 and the end of amphotericin therapy. Individuals who died before the end of amphotericin therapy, and in whom no negative cultures were observed prior to death, were classified into the death group.

Twenty-three individuals (13% of 177) did not have any negative cultures during therapy and also did not have a culture after day 12 of induction therapy, despite surviving, leaving the sterility outcome unobserved. Reasons why an LP was not done at the end of amphotericin were not always documented, however, a common reason was that the patient refused another LP. The primary analysis was restricted to the 154 inidivuals with observed outcomes, excluding these 23 individuals; however, sensitivity analysis of the statistical models were conducted using multiple imputation to estimate whether sterility would have been observed in these 23 individuals.

The exact timing of sterility could not be determined for any patient as CSF cultures were conducted only after a lumbar puncture (LP) and no individuals had daily LPs; however, rough estimates of the timing could made based on sampling periods during which LPs were performed. From these sampling periods, sterility was further defined as early or late during induction therapy, according to the timing of the first observed sterile culture, with early sterility defined as sterility observed before day 12 of amphotericin therapy and late sterility defined as sterility observed between day 12 and the end of amphotericin therapy.

4.2.3 CSF Cryptococcal Antigen Measurement

Semi-quantitative cryptococcal antigen (CRAG) titers were measured in cryopreserved CSF samples stored at the time of CM diagnosis. The CRAG titer was measured at the University of Minnesota using the CRAG lateral flow assay (LFA; IMMY Inc., Norman, OK). Two-fold serial dilutions were used to estimate the CRAG titer. Semi-quantitative titers were $\log_2$ transformed for analysis.

4.2.4 Early Fungicidal Activity

The rate of cryptococcal clearance from the CSF, also termed the early fungicidal activity or EFA [29], was calculated from the first observed quantitative culture to either the first negative culture, the end of amphotericin therapy, or death, whichever occurred first (see Appendix B, section B.1). Subject-specific linear regression analysis was conducted using $\log_{10}$ CFU/mL as the dependent variable and days of amphotericin as the independent variable. The slope of the regression equation is the estimated EFA for a particular individual. The EFA over two weeks and the EFA during the first week of amphotericin (up to day 11) were both calculated and evaluated for their association with CSF sterility. The EFA times -1 was used in analysis such that a higher positive value meant a more rapid decline in cryptococcal cells from the CSF.

4.2.5 Statistical Analysis

Recognizing that death could be a competing risk for observing a sterile CSF, analyses were initially conducted using a multi-level outcome defined by CSF sterility, CSF non-sterility, or death. These analyses were conducted using polytomous logistic regression with CSF sterility as the common reference. Because the polytomous regresssion models did not identify factors solely related to mortality, and the size of the mortality group was

relatively small, the primary focus of further analysis was on distinguishing individuals with a sterile versus non-sterile CSF by the end of therapy among those who survived to the end of amphotericin therapy using binary logistic regression.

Initial exploration of covariates possibly associated with CSF sterility was conducted using Wilcoxon ranksum tests to compare medians and χ^2 tests to compare frequencies. Covariates demonstrating a difference between the sterility groups with a p-value $\leq$0.10, were explored in univariate logistic regression with CSF sterility compared to non-sterility. A final multivariable model of sterility was reached using backwards elimination from a full model including all covariates univariately associated with sterility with a p-value $\leq$ 0.10. The same final model was also reached using a stepwise model-building approach (results not shown). Cryptococcal quantitative culture and CRAG titer were not included in models together due to their strong correlation (Pearson correlation coefficient = 0.59, p-value <0.001); therefore, final models are presented for quantitative culture and CRAG titer, separately.

A C statistic was estimated to assess the prediction of each model. The C statistic is a measure of model prediction describing the probability that the model-estimated odds of the outcome, among those with the outcome, is greater than the model-estimated odds in those without the outcome. This is equivalent to the area-under-the-curve. C statistic values close to 0.5 indicate model prediction is no better than chance, whereas values above 0.8 suggest models that have strong prediction.

Because CSF sterility was not a rare outcome, the odds ratios calculated from regression coefficients are less equivalent to the observed risk of sterility. Therefore, predicted marginal probabilities of sterility were estimated to better describe the relationship with baseline characteristics. These probabilities were estimated from regression formulas using Stata version 12 (StataCorp, College Station, TX).

Finally, cryptococcal burden cut-points were created from the baseline quantitative

cultures and CSF CRAG titers to assess the ability of these measures to correctly identify patients who would have sterile CSF by the end of two weeks of amphotericin, as they may be candidates for a shortened course of amphotericin. Sensitivity, specificity, and positive predictive values were calculated for each cut-point. Sensitivity was defined as the probability of a patient being below the cut-point given that the patient reached CSF sterility. Likewise, the specficity was defined as the probability that a patient was above the cut-point given that the patient did not reach CSF sterility. Positive predictive value was defined as the probability that a patient who did reach CSF sterility had a baseline fungal burden below the cut-point. The optimal cut-point was chosen based on maximizing specificity while keeping sensitivity as high as possible. Specificity was chosen as the metric to optimize because it would be advantageous to reduce the proportion of false positives, or patients who would be falsely identified as candidates for shortened therapy. In addition to sensitivity, specificity, and positive predictive value, the proportion of individuals with mid-treatment cultures that were sterile and mid-treatment cultures that were 100 CFU/mL or less was compared for each cut-point. Mid-treatment cultures were cultures taken between day 5 and 11 of amphotericin treatment. This time period was chosen to capture as many individuals as possible.

Data processing and analysis, aside from model-predicted probabilities of CSF sterility, were conducted using SAS version 9.3 (SAS Institute, Cary, NC). Statistical significance was considered for all associations with p-values <0.05.

4.2.6 Missing Data

Sensitivity analyses was conducted to assess the robustness of the findings to missing baseline covariates and unobserved sterilty outcomes. The most common missing baseline measure was weight (missing for 31% of participants), due to the inability of many of the individuals to stand on their own for effective measurement. Other common missing

factors were the 1-week EFA (18% missing), CSF opening pressure (14% missing), and serum potassium (12% missing). All other variables were missing in less than 6% of the sample. Reasons for missing data stemmed from laboratory contamination or insufficient measurements, supplies, or sample volumes to get valid data. As such, missing data were assumed to be missing at random; that is, the likelihood of a parameter being missing was not thought to be dependent on the unobserved value of the parameter [84]. Body weight had the potential to be missing not at random, as those missing weight likely had more severe disease. Because of this an indicator for missing weight status was investigated for its association with sterility status to assess whether missing weight could be a potential confounder.

For multiple imputation, a Markov Chain Monte-Carlo process was used and 40 full datasets were imputed. The following baseline parameters were included in the multiple imputation model: age, sex, and CSF parameters over induction therapy including opening pressures, quantitative cultures, white blood cell counts, and days of amphotericin. This model was used to impute both missing baseline measures as well as missing sterility outcomes. Of the 40 datasets, 1 model predicted 81 sterile and 80 non-sterile individuals whereas 5 models predicted 82 and 79, 13 models predicted 83 and 78, 10 models predicted 84 and 77, 3 models predicted 85 and 76, 3 models predicted 86 and 75, and 5 models predicted 87 and 74 sterile and non-sterile individuals, respectively.

4.3 Results

4.3.1 Study Population

Table 4.1: Baseline demographic and clinical characteristics by sterility status at the end of amphotericin therapy for individuals with cryptococcal meningitis in the COAT trial.

	Total subjects		Died before 2 weeks		Unknown sterility		Sterile by 2 weeks		Not sterile at 2 weeks		P-value [a]
N per group	177		16 (9%)		23 (13%)		81 (46%)		57 (32%)		
Study site [b]											0.53
Kampala	115		15 (13%)		13 (11%)		48 (42%)		39 (34%)		
Mbarara	35		1 (3%)		7 (20%)		17 (49%)		10 (29%)		
Cape Town	27		0 (0%)		3 (11%)		16 (59%)		8 (30%)		
	N with data	Median [IQR] [c]	N with data	Median [IQR] [c]	N with data	Median [IQR] [c]	N with data	Median [IQR] [c]	N with data	Median [IQR] [c]	
Treatment group, N (%)	177		16		23		81		57		0.65
Early ART		88 (50%)		8 (50%)		13 (57%)		42 (52%)		25 (44%)	
Deferred ART		89 (50%)		8 (50%)		10 (44%)		39 (48%)		32 (56%)	
Age (years)	177	35 [29, 40]	16	38 [32, 40]	23	35 [27, 38]	81	34 [27, 40]	57	37 [30, 42]	0.09
Males, N (%)	177	93 (53%)	16	5 (31%)	23	12 (52%)	81	38 (47%)	57	38 (67%)	0.02
Weight (kg)	124	54.0 [46.0, 59.2]	11	50.0 [45.0, 55.0]	18	54.0 [48.8, 59.3]	53	54.0 [46.0, 59.0]	42	54.5 [47.0, 62.0]	0.52
Weight missing	177	53 (30%)	16	5 (31%)	23	5 (22%)	81	28 (35%)	57	15 (26%)	0.30
Headache duration	172		16		23		77		56		0.32
less than 7 days		18 (11%)		1 (6%)		6 (26%)		6 (8%)		5 (9%)	
7-13 days		56 (33%)		2 (13%)		6 (26%)		31 (40%)		17 (30%)	
14-20 days		35 (20%)		5 (31%)		1 (4%)		17 (22%)		12 (21%)	
21-28 days		19 (11%)		1 (6%)		3 (13%)		5 (7%)		10 (18%)	
28 days or more		44 (26%)		7 (44%)		7 (30%)		18 (23%)		12 (21%)	
Karnofsky Score	176	50 [40, 60]	16	50 [45, 50]	23	50 [40, 60]	81	50 [40, 50]	56	50 [40, 50]	0.84
Glasgow Coma Scale	176		16		23		81		56		0.20
<15		47 (27%)		6 (38%)		1 (4%)		27 (33%)		13 (23%)	
15		129 (73%)		10 (63%)		22 (96%)		54 (67%)		43 (77%)	
Heart rate (beats per minute)	175	80 [69, 90]	15	84 [71, 93]	23	76 [66, 83]	81	80 [71, 90]	56	76 [64, 90]	0.17

Continued on next page

Table 4.1 – continued from previous page

	Total subjects		Died before 2 weeks		Unknown sterility		Sterile by 2 weeks		Not sterile at 2 weeks		P-value [a]
Respiratory rate (breaths per minute)	173	20 [20, 24]	15	22 [20, 24]	23	20 [19, 22]	80	22 [20, 24]	55	20 [19, 24]	0.13
Systolic blood pressure (mmHg)	173	110 [106, 122]	15	120 [100, 129]	23	108 [100, 117]	79	113 [108, 123]	56	110 [106, 120]	0.27
Diastolic blood pressure (mmHg)	173	70 [60, 80]	15	70 [60, 80]	23	61 [60, 74]	79	70 [60, 83]	56	70 [61, 82]	0.71
Axillary temperature (^oC)	174	36.6 [36.0, 37.2]	15	36.2 [36.0, 36.6]	22	36.8 [36.1, 37.5]	81	36.8 [36.2, 37.7]	56	36.3 [35.8, 36.8]	0.004
Fever (axillary temperature >37.5^oC)	174	35 (20%)	15	0 (0%)	22	5 (23%)	81	23 (28%)	56	7 (13%)	0.03
Clinical Laboratory Values											
Hemoglobin (g/dL)	173	11.2 [9.1, 12.9]	15	10.5 [8.9, 12.4]	23	10.8 [9.3, 12.2]	79	10.6 [8.6, 13.1]	56	11.7 [10.1, 13.2]	0.05
Hematocrit (%)	173	32.8 [27.3, 37.9]	15	30.7 [26.9, 36.8]	23	30.6 [24.5, 37.0]	79	31.4 [25.9, 38.2]	56	34.6 [29.6, 38.9]	0.09
White blood cells (x$10^3/\mu$L)	173	3.4 [2.6, 5.2]	15	3.4 [2.7, 4.6]	23	3.3 [2.8, 5.2]	79	3.6 [2.5, 5.4]	56	3.4 [2.5, 5.1]	0.86
Creatinine (mg/dL)	172	0.7 [0.5, 0.9]	15	0.7 [0.6, 0.8]	23	0.7 [0.5, 0.8]	79	0.7 [0.5, 0.9]	55	0.6 [0.5, 0.9]	0.90
Potassium (mmol/L)	157	3.9 [3.4, 4.3]	14	4.0 [3.2, 4.1]	22	3.8 [3.5, 4.4]	70	3.9 [3.4, 4.2]	51	4.0 [3.4, 4.3]	0.64
CSF Parameters [d]											
Opening pressure (mmH_2O)	152	269 [180, 378]	16	225 [180, 300]	20	233 [175, 363]	64	260 [175, 355]	52	305 [215, 437]	0.05
Opening pressure > 250 mmH_2O, N (%)	152	87 (57%)	16	8 (50%)	20	9 (45%)	64	35 (55%)	52	35 (67%)	0.17
Quantitative cryptococcal culture ($\log_{10}$ CFU/mL)	168	5.1 [4.0, 5.6]	16	5.3 [4.7, 5.4]	23	5.3 [4.0, 5.8]	76	4.5 [3.0, 5.4]	53	5.4 [4.7, 5.8]	<0.001
Cryptococcal antigen titer (1:x)	169	2560 [640, 8000]	13	4000 [1000, 8192]	23	2000 [1024, 16000]	78	2000 [400, 4000]	55	4096 [1280, 12800]	0.006
White blood cells (/μL)	166	15 [<5, 105]	15	30 [<5, 50]	21	8 [<5, 40]	77	69 [<5, 165]	53	7 [<5, 31]	0.002
White blood cells < 5/μL	166	63 (38%)	15	7 (47%)	21	9 (43%)	77	22 (29%)	53	25 (47%)	0.03
Randomization HIV Parameters											
CD4 count (cells/μL)	175	23 [10, 74]	14	17 [13, 42]	23	36 [10, 75]	81	38 [10, 78]	57	17 [8, 70]	0.09

Continued on next page

Table 4.1 – continued from previous page

	Total subjects		Died before 2 weeks		Unknown sterility		Sterile by 2 weeks		Not sterile at 2 weeks		P-value [a]
HIV viral load (log_{10} copies/mL)	175	5.5 [5.2, 5.8]	15	5.5 [5.4, 5.8]	23	5.8 [5.4, 5.9]	80	5.5 [5.2, 5.8]	57	5.5 [5.1, 5.7]	0.60
Changes in Parameters over time											
Early fungicidal activity, day 0 to 11 (log_{10} CFU/mL/day)	145	0.38 [0.23, 0.53]	14	0.29 [0.19, 0.40]	17	0.35 [0.16, 0.43]	71	0.45 [0.32, 0.62]	43	0.34 [0.18, 0.46]	0.003
Early fungicidal activity, day 0 to 14 (log_{10} CFU/mL/day)	166	0.32 [0.23, 0.41]	14	0.30 [0.19, 0.36]	17	0.35 [0.16, 0.43]	78	0.39 [0.33, 0.56]	57	0.24 [0.18, 0.30]	<0.001

[a] P-values from chi-square test for frequencies and Wilcoxon ranksum test for medians comparing the sterile group to the non-sterile group.

[b] Proportion of each site within the sterility group.

[c] Medians with 25^{th} percentile to 75^{th} percentile, interquartile range (IRQ); unless otherwise noted.

[d] CSF parameters from lumbar punctures performed at the time of CM diagnosis.

One hundred seventy-seven individuals with HIV and a first episode of CM were enrolled in the COAT trial. Twenty-three (13%) individuals did not have a CSF culture after the 12^{th} day of amphotericin induction therapy with which to assess sterility, leaving 154 individuals available for the primary analysis, including 13 individuals who died prior to sterility being observed. The 154 individuals in this analysis were very similar to all participants in the COAT trial and other described cohorts of CM patients in sub-Saharan Africa [43, 57–59]. The median age of the individuals in this analysis was 36 years, 53% were males, 30% had altered mental status (Glasgow Coma Scale <15) at the time of diagnosis, and individuals had experienced a headache for a median of two weeks prior to CM diagnosis (table 4.1). As is typical with HIV-associated CM, the 154 participants had severe immunosuppression with a median CD4 count of 21 cells/μL and median HIV viral load greater than 300,000 copies/mL. Ninty percent of patients received between 12 and 15 days of amphotericin therapy.

4.3.2 Participant Outcomes

During induction therapy, participants underwent a median of 3 cultures (9 (5%) with 1 culture, 45 (25%) with 2, 82 (46%) with 3, 31 (18%) with 4, 9 (5%) with 5, and 1 (1%) with 6 cultures) from which CSF sterility could be assessed. Of all participants, 16 (9%) individuals died before the end of treatment and with no observed negative cultures, 57 (32%) individuals failed to reach CSF sterility, and 81 (46%) were found to have sterile CSF by the end of amphotericin (figure 4.1). Of the 81 with sterile CSF cultures, 40 individuals (49%) had sterile CSF before the 12^{th} day of amphotericin and 41 individuals (51%) had sterile CSF observed after day 12 of amphotericin.

Among those found to have non-sterile CSF at the end of therapy, the median fungal burden was 100 CFU/mL (25^{th} to 75^{th} percentile: 20 to 500 CFU/mL; figure 4.2) at the end of amphotericin and 12 individuals (8%) had final fungal burdens greater than 1,000

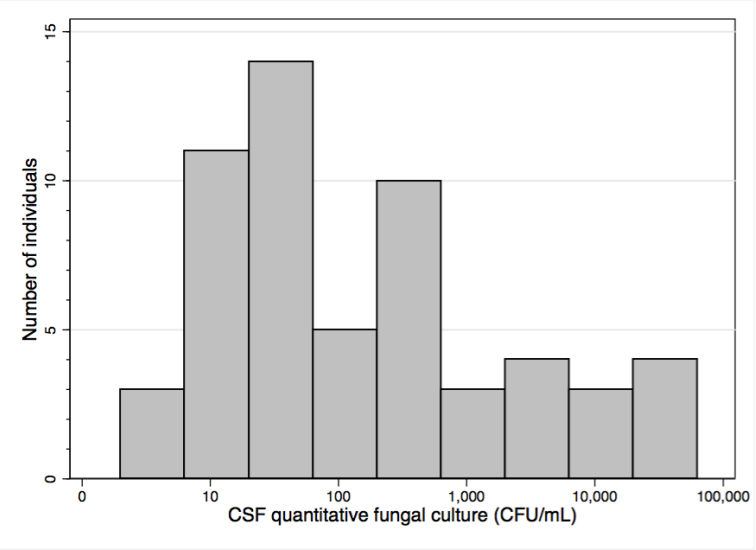


Figure 4.2: Distribution of fungal burden at the end of amphotericin therapy among individuals with cryptococcal meningitis who did not reach CSF sterility in the COAT trial.

CFU/mL at the end of amphotericin therapy.

4.3.3 Univariate Associations

Sterility by the end of therapy was found to be univariately related, in logistic regression analysis, to being female and the following baseline clinical factors, higher body temperature, lower baseline CSF quantitative culture, lower CSF cryptococcal antigen (CRAG) titer, and a faster early fungicidal activity (EFA) during the first week, as well as throughout two weeks of therapy (table 4.2). As the focus of this analysis was to predict which patients were more likely to reach sterility, only the EFA up through the first week of amphotericin was taken forward into multivariable models. The median absolute change in quantitative fungal burden over the course of amphotericin therapy was also substantially higher, at 24,800 CFU/mL (25^{th} to 75^{th} percentile: 808 to 205,500 CFU/mL), among those who reached CSF sterility compared to 1,272 CFU/mL among those who did not reach sterility (25^{th} to 75^{th} percentile: 172 to 6,882 CFU/mL). CSF sterility was not found

Table 4.2: Univariate logistic regression associations with sterile CSF versus non-sterile CSF by the end of amphotericin therapy among individuals with cryptococcal meningitis in the COAT trial.

Covariate	N	OR (95% CI) [a]	P-value
Age (years)	138	0.97 (0.93, 1.01)	0.11
Males (versus females)	138	0.44 (0.22, 0.89)	0.02
Axillary temperature (^{o}C)	137	1.67 (1.17, 2.39)	0.01
Fever (axillary temperature >37.5^{o}C)	137	2.77 (1.10, 7.14)	0.03
Hemoglobin (g/dL)	135	0.88 (0.77, 1.01)	0.07
Hematocrit (%)	135	0.96 (0.92, 1.01)	0.10
CD4 count (cells/μL)	138	1.01 (1.00, 1.01)	0.06
CSF opening pressure (mmH_2O)	116	0.97 (0.95, 1.00)	0.04
CSF opening pressure $\geq$ 250 mmH_2O	116	0.59 (0.27, 1.25)	0.17
CSF white blood cells (/10 μL)	130	1.06 (1.02, 1.10)	0.005
CSF quantitative cryptococcal culture ($\log_{10}$ CFU/mL)	129	0.54 (0.39, 0.75)	<0.001
CSF cryptocccal antigen titer ($\log_2$ 1:titer)	133	0.82 (0.71, 0.96)	0.01
Early fungicidal activity, over 1 week (x10 $\log_{10}$ CFU/mL/day)	114	1.30 (1.08, 1.56)	0.005
Early fungicidal activity, over 2 weeks (x10 $\log_{10}$ CFU/mL/day)	135	3.21 (2.05, 5.04)	<0.001

[a] Odds ratio (OR) and 95% confidence interval (CI)

to be associated with COAT treatment arm (52% of the early arm compared to 48% of the deferred arm has sterile CSF, p-value = 0.56), therefore multivariable analysis did not further adjust for treatment arm. The median days of amphotericin-based therapy also did not differ between sterility groups (median of 14 days in both groups, 25^{th} to 75^{th} percentile: 14 - 14).

4.3.4 Multivariable Associations

Table 4.3: Multivariable logistic regression of characteristics related to sterile versus non-sterile CSF by the end of amphotericin therapy among participants in the COAT trial.

	Full Model	Reduced Model 1	Reduced Model 2	Reduced Model 3	Reduced Model 4	Reduced Model 5	Reduced Model 6
Observations in the model	91	91	106	106	111	111	112
Number reaching sterility (%)	53 (58%)	53 (58%)	66 (62%)	66 (62%)	69 (62%)	69 (62%)	70 (63%)
Number not reaching sterility (%)	38 (42%)	38 (42%)	40 (38%)	40 (38%)	42 (38%)	42 (38%)	42 (37%)
Model C statistic [a]	0.820	0.821	0.810	0.813	0.817	0.803	0.787
	OR (95%CI) [b]	OR (95%CI)	OR (95%CI)	OR (95%CI)	OR (95%CI)	OR (95%CI)	OR (95%CI)
CSF quantitative cryptococcal culture ($\log_{10}$ CFU/mL)	0.61 (0.36, 1.04)	0.59 (0.36, 0.97)	0.59 (0.38, 0.92)	0.58 (0.37, 0.91)	0.51 (0.34, 0.77)	0.49 (0.33, 0.74)	0.47 (0.32, 0.70)
Early fungicidal activity, day 0 to 11 (x10 $\log_{10}$ CFU/mLday)	1.26 (1.01, 1.58)	1.27 (1.01, 1.58)	1.30 (1.03, 1.63)	1.33 (1.06, 1.68)	1.40 (1.11, 1.78)	1.48 (1.17, 1.86)	1.41 (1.14, 1.74)
Hemoglobin (g/dL)	0.84 (0.67, 1.04)	0.83 (0.67, 1.03)	0.82 (0.66, 1.01)	0.81 (0.65, 1.00)	0.79 (0.65, 0.97)	0.81 (0.66, 0.98)	
Axillary temperature (^{o}C)	1.51 (0.89, 2.54)	1.53 (0.91, 2.56)	1.65 (1.01, 2.70)	1.55 (0.96, 2.50)	1.58 (0.99, 2.53)		
CSF white blood cells (/10 μL)	1.04 (0.98, 1.09)	1.04 (0.99, 1.09)	1.03 (0.98, 1.09)	1.03 (0.98, 1.09)			
Males (versus females)	0.57 (0.19, 1.74)	0.57 (0.19, 1.73)	0.57 (0.20, 1.59)				
CSF opening pressure $\geq$250 mmH_2O	0.72 (0.23, 2.28)	0.74 (0.23, 2.31)					
CD4 count (cells/μL)	1.00 (0.99, 1.01)						

[a] Model C statistic is a measure of model prediction describing the probability that the model-estimated odds of the outcome, among those with the outcome, is greater than the model-estimated odds in those without the outcome. This is equivalent to the area-under-the-curve. Values close to 0.5 indicate model prediction no better than chance, whereas values above 0.8 suggest models with strong prediction.

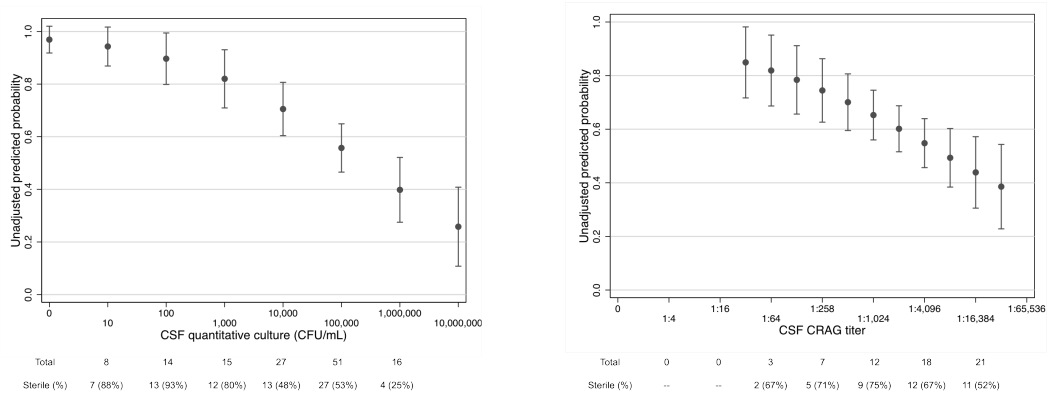
[b] Odds ratio (OR) and 95% confidence interval (CI)

In multivariable analysis, using backwards elimination of non-significant covariates, the only remaining baseline clinical or demographic factors independently associated with CSF sterility were lower baseline quantitative culture, faster 1-week EFA, and baseline hemoglobin (table 4.3, reduced model 5). A further reduced model, excluding baseline hemoglobin, demonstrated slightly lower prdictive ability but similar estimates of the odds of sterility for increases in quantitative culture and 1-week EFA (reduced model 6).

Analysis after multiple imputation of the unobserved sterility outcome did not result in a different final models, nor in materially different parameter estimates (results from reduced model 5: quantitative culture OR = 0.48, 95% CI: 0.34, 0.69; 1-week EFA OR = 1.41, 95% CI: 1.14, 1.74; and hemoglobin OR = 0.83, 95% CI: 0.70, 0.99). Similarly, multiple imputation of the baseline clinical characteristics did not lead to a different final model or different parameter estimates (results from reduced model 5: quantitative culture OR = 0.48, 95% CI: 0.34, 0.69; 1-week EFA OR = 1.41, 95% CI: 1.13, 1.74; and hemoglobin OR = 0.81, 95% CI: 0.66, 0.98).

In models considering the CSF CRAG titer rather than the quantitative culture, similar results were found and a final reduced model included the baseline CRAG titer, hemoglobin, and the 1-week EFA. Each doubling of the CSF CRAG titer was associated with a 17% reduction in the odds of sterility (OR = 083, 95% CI: 0.69, 0.98), a 29% increase in odds of sterility with each 0.10 increase in 1-week EFA (OR = 1.29, 95% CI: 1.07, 1.57), and an 18% decrease with each g/dL increase in baseline hemoglobin (OR = 0.82, 95% CI: 0.69, 0.83); the overall model C statistic was 0.75. Excluding hemoglobin from the model, again, result in a reduction in the model C statistic (0.73) and slight changes in the 1-week EFA odds ratio (OR = 1.37, 95% CI: 1.07, 1.58; CRAG OR = 0.81, 95% CI: 0.68, 0.95).

As the prevalence of a sterile CSF culture was fairly common in this cohort, occurring in 47% of those randomized, the odds of CSF sterility are less reflective of the probability of sterility. Therefore, figure 4.3 depicts the predicted probabilities, or risk, of CSF sterility



(a) (b)

Figure 4.3: Predicted probability of CSF sterility by the end of amphotericin therapy for baseline CSF (a) quantitative culture and (b) cryptococcal antigen (CRAG) titer, from unadjusted logistic regression, for individuals with cryptococcal meningitis in the COAT trial. Observed probabilities are calculated for subjects with baseline quantitative culture values between the major axis labels, for example the observed sterility for subjects with baseline cultures between 101 and 1,000 CFU/ml. Observed probabilities are calculated for baseline CRAG titers for subjects with titers at each axis label, some CRAG titers have been omitted for clarity.

from crude models with CSF fungal burden. As the logistic models indicated, the probability of CSF sterility increases with decreasing baseline quantitative culture and CRAG titer. Individuals with fungal burdens lower than 1,000 CFU/mL, from quantitative culture, or CSF CRAG titer of less than 1:64 had a predicted probability of sterility greater than 80%. The predicted probabilities changed very little after adjustment for the EFA in the first week and baseline hemoglobin (figure 4.4).

4.3.5 Participant Profiles for Prediction

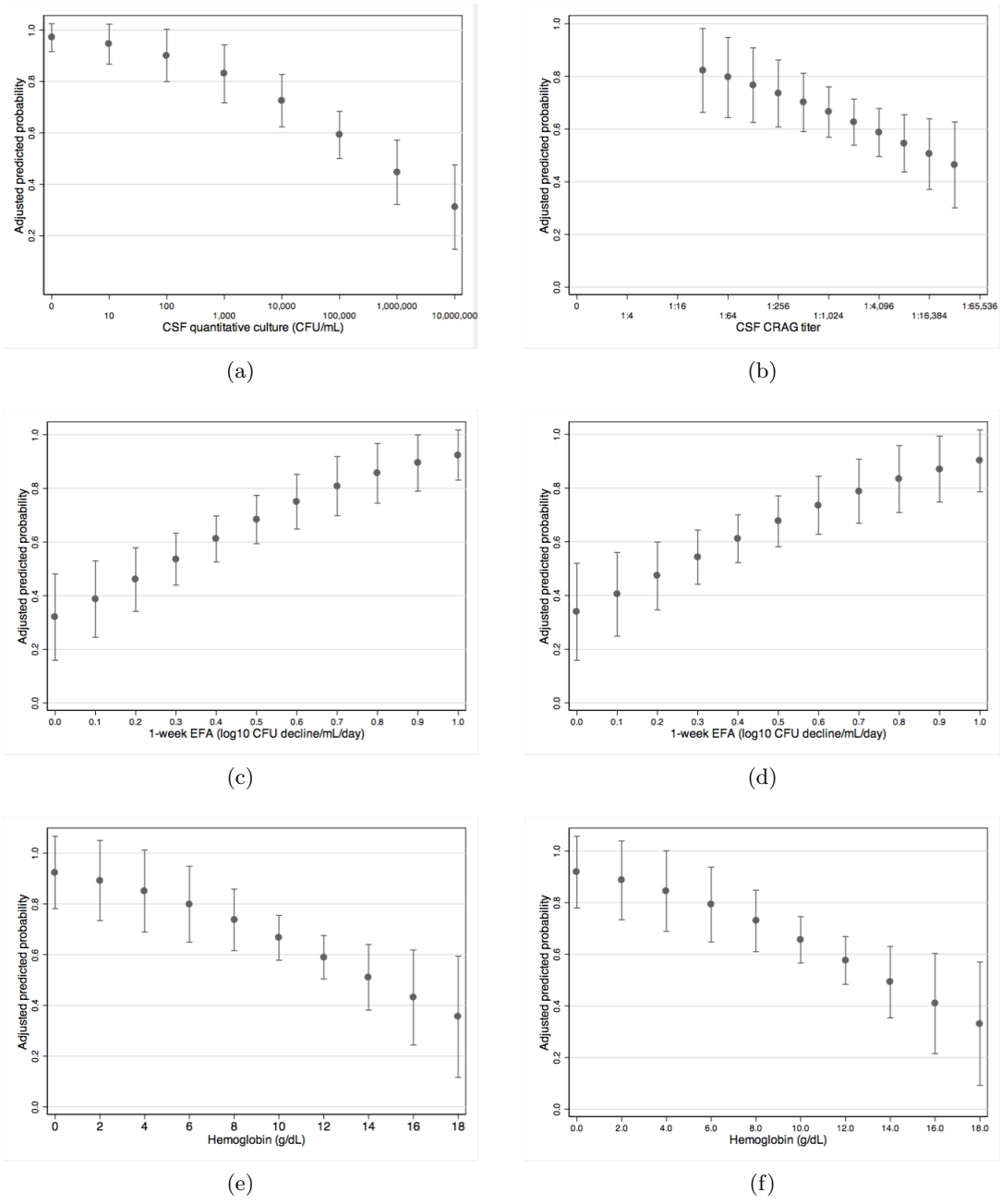


Figure 4.4: Adjusted predicted probability of CSF sterility by the end of amphotericin therapy for individualswith cryptococcal meningitis in the COAT trial; (a) predicted probability of CSF sterility by baseline CSF quantitative culture, adjusted for 1-week early fungicidal activity (EFA) and baseline hemoglobin, (b) predicted probability of CSF sterility by baseline CSF cryptococcal antigen (CRAG) titer, adjusted for 1-week EFA and baseline hemoglobin, (c) predicted probability of CSF sterility by 1-week EFA, adjusted for baseline CSF quantitative culture burden and hemoglobin, (d) predicted probability of CSF sterility by 1-week EFA, adjusted for baseline CSF CRAG titer and baseline hemoglobin, (e) baseline hemoglobin, adjusted for baseline CSF quantitative culture burden and 1-week EFA, and (f) baseline hemoglobin, adjusted for baseline CSF CRAG titer and 1-week EFA.

Table 4.4: Fungal burden thresholds for predicting CSF sterility at the end of amphotericin therapy among participants in the COAT trial.

	Sterile below cut-point, N(%)	Non-sterile below cut-point, N(%)	Sterile above cut-point, N(%)	Non-sterile above cut-point, N(%)	Sensitivity [a]	Specificity [a]	Positive Predictive Value [a]	N with mid-treatment culture [b]	Mid-treatment culture ≤100 CFU/mL, N(%) [c]	Sterile mid-treatment culture, N(%) [d]
CSF Quantitative culture (CFU/mL) [e]										
≤ 100	7	1	69	52	9%	98%	88%	7	7 (100%)	5 (71%)
≤ 1,000	20	2	56	51	26%	96%	91%	19	18 (95%)	15 (79%)
≤ 10,000	32	5	44	48	42%	91%	86%	32	30 (94%)	22 (69%)
≤ 100,000	45	19	31	34	59%	64%	70%	57	41 (72%)	25 (44%)
≤ 1,000,000	72	42	4	11	95%	21%	63%	103	60 (58%)	33 (32%)
CSF CRAG titer [f]										
≤ 1:128	5	1	73	54	6%	98%	83%	5	5 (100%)	4 (80%)
≤ 1:256	15	6	63	49	19%	89%	71%	16	12 (75%)	8 (50%)
≤ 1:512	23	10	55	45	29%	82%	70%	27	19 (70%)	11 (41%)
≤ 1:1,024	31	13	47	42	40%	76%	70%	38	27 (71%)	17 (45%)
≤ 1:2,048	44	20	34	35	56%	64%	69%	55	39 (71%)	24 (44%)
≤ 1:4,096	59	27	19	28	76%	51%	69%	74	52 (70%)	32 (43%)
≤ 1:8,192	68	41	10	14	87%	25%	62%	96	59 (61%)	33 (34%)
≤ 1:16,384	74	47	4	8	95%	15%	61%	108	64 (59%)	33 (31%)
≤ 1:32,768	77	55	1	0	99%	0%	58%	119	65 (55%)	33 (28%)

[a] Sensitivity was calculated as the probability of being below the cut-point given the outcome was a sterile CSF culture. Specificity was calculated as the probability of being above the cut-point given the outcome was a non-sterile CSF culture. Positive predictive value was calculated as the probability of a sterile CSF culture given the baseline burden was below the cut-point.

[b] Mid-treatment was defined as cultures observed between days 5 and 11 of amphotericin.

[c] The proportion of individuals with mid-treatment quantitative culture <100 CFU/mL, among those with mid-treatment cultures and baseline burdens below the cut-point.

[d] The proportion of individuals with a sterile mid-treatment quantitative culture, among those with mid-treatment cultures and baseline burdens below the cut-point.

[e] Nine individuals were missing baseline quantitative cultures.

[f] Five individuals were missing baseline cryptococcal antigen titers.

Practically speaking, the 1-week EFA could be difficult to measure in a timely manner in many settings given that growth of *C. neoformans* can be slow. Univariately, baseline CSF fungal burden was less predictive of sterility (model C statistic of 0.69 for quantitative culture) than a model that also incorportated the 1-week EFA and baseline hemoglobin; however, the baseline fungal burden could be readily available in many places and possibly used independently to inform choices about the duration of amphotericin therapy. Various cut-points of quantitative culture and CSF CRAG titer were examined for their use as means to differentiate individuals who did and did not reach sterility by the end of two weeks of amphotericin therapy (table 4.4). Based on wanting to maximize specificity, perhaps the best cut-point for classifying individuals in this cohort was a baseline fungal burden of 10,000 CFU/mL. The positive predictive value of this cut-point, in the setting of the COAT trial, indicated that 86% of individuals with fungal burden 10,000 CFU/mL or less had reached a sterile CSF culture by the end of induction therapy. Higher cut-points had vastly reduced specificity and, thus, lower positive predictive values. Also, at the 10,000 CFU/mL cut-point, 69% of participants with baseline fungal burdens at or below the threshold had a sterile culture when assessed between 5 and 11 days after CM diagnosis and 94% had a fungal burden of 100 CFU/mL or lower at this mid-treatment timepoint, suggesting that one more week of amphotericin may not have been necessary.

In general, it appeared that the specificity of a cut-point based on CSF CRAG titers was lower than when using CSF quantitative culture; this was also shown by a lower univariate model C statistic with baseline CRAG (0.64). However, the CSF CRAG titer may be easier to obtain in rural settings and primary care clinics, and CRAG titers will also be available well before CSF culture results as the LFA platform requires only a 15 minute incubation period. Within this cohort, using a titer cut-point of 1:512 resulted in a specificity of 82%, but a low sensitivity of 29%. The specificity decreased and sensitivity increased with a higher cut-point of 1:1024, and without a decline in the positive predictive

value. Thus, a cut-point of 1:1024 may be most suitable. Of note, with a 1:1024 threshold, 45% of individuals at or below the cut-point had sterile culture and 71% had a culture with 100 CFU/mL or less at the mid-point of amphotericin therapy. These proportions did not differ substantially when the cut-point was lowered to a titer of 1:512.

4.3.6 Early versus Late Sterility

Fourty-two (24% of 177) individuals in the COAT trial reached CSF sterility before the 12^{th} day of amphotericin induction therapy. Exploratory univariate analyses were conducted to see what variables distinguished those reaching sterility early on during amphotericin therapy from those reaching sterility by the end of therapy.

Table 4.5: Baseline demographic and clinical characteristics for individuals with cryptococcal meningitis in the COAT trial who reached CSF sterility by end of amphotericin therapy.

	Sterile before day 12		**Sterile after day 12**		
N per group [a]	40 (49%)		41 (51%)		
	N with data	Median [IQR] [b]	N with data	Median [IQR] [b]	P-value [c]
Treatment Group, N (%)	40		41		0.44
Early ART		19 (48%)		23 (56%)	
Deferred ART		21 (53%)		18 (44%)	
Age (years)	40	34 [27, 42]	41	33 [27, 40]	0.64
Males, N (%)	40	13 (33%)	41	25 (61%)	0.01
Weight (kg)	28	55.0 [46.3, 61.3]	25	52.5 [46.0, 58.0]	0.65
Weight missing		12 (30%)		16 (39%)	0.40
Headache duration	38		39		0.50
less than 7 days		2 (5%)		4 (10%)	
7-13 days		15 (39%)		16 (41%)	
14-20 days		9 (24%)		8 (21%)	
21-28 days		1 (3%)		4 (10%)	
28 days or more		11 (29%)		7 (18%)	

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Table 4.5 – continued from previous page

	Sterile before day 12		Sterile after day 12		
Karnofsky Score	40	50 [40, 50]	41	50 [40, 60]	0.61
Glasgow Coma Scale	40		41		0.88
<15		13 (33%)		14 (34%)	
15		27 (68%)		27 (66%)	
Heart rate (beats per minute)	40	80 [68, 89]	41	80 [72, 93]	0.33
Respiratory rate (breaths per minute)	39	22 [20, 24]	41	22 [20, 24]	0.65
Systolic blood pressure (mmHg)	39	110 [108, 123]	40	114 [109, 128]	0.57
Diastolic blood pressure (mmHg)	39	70 [60, 80]	40	75 [60, 85]	0.41
Axillary remperature (^{o}C)	40	37.0 [36.5, 38.0]	41	36.7 [35.9, 37.3]	0.05
Fever (axillary temperature >37.5^{o}C)	40	17 (43%)	41	6 (15%)	0.006
Clinical Laboratory Values					
Hemoglobin (g/dL)	38	11.1 [8.9, 13.3]	41	10.5 [8.4, 11.9]	0.51
Hematocrit (%)	38	33.2 [26.0, 39.8]	41	30.8 [25.9, 35.7]	0.50
White blood cells (x$10^3/\mu$L)	38	4 [3, 6]	41	3 [2, 5]	0.30
Creatinine (mg/dL)	38	0.7 [0.5, 0.9]	41	0.7 [0.5, 0.9]	0.71
Potassium (mmol/L)	31	4.0 [3.7, 4.3]	39	3.7 [3.3, 4.1]	0.07
CSF Parameters [d]					
Opening pressure (mmH_2O)	31	240 [150, 310]	33	290 [210, 410]	0.07
Opening pressure > 250 mmH_2O, N (%)	31	14 (45%)	33	21 (64%)	0.14
Quantitative cryptococcal culture ($\log_{10}$ CFU/mL)	37	3.1 [2.7, 5.2]	39	5.1 [4.4, 5.5]	0.002
Cryptococcal antigen titer (1:titer)	39	1000 [250, 2560]	39	4000 [640, 8000]	0.007
White blood cells (/μL)	38	125 [33, 350]	39	5 [<5, 80]	<0.001
White blood cells < 5/μL	38	4 (11%)	39	18 (46%)	0.001

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Table 4.5 – continued from previous page

	Sterile before day 12		Sterile after day 12		
HIV Parameters					
CD4 count (cells/μL)	40	63 [17, 112]	41	19 [9, 51]	0.009
HIV viral load ($\log_{10}$ copies/mL)	40	5.4 [5.1, 5.9]	40	5.5 [5.2, 5.8]	0.98
Changes in Parameters Over Time					
Early fungicidal activity, day 0 to 11 ($\log_{10}$ CFU/mL/day)	37	0.58 [0.39, 0.69]	34	0.41 [0.23, 0.49]	0.003
Early fungicidal activity, day 0 to 14 ($\log_{10}$ CFU/mL/day)	37	0.58 [0.39, 0.69]	41	0.35 [0.32, 0.39]	<0.001

[a] Among the 81 COAT participants with observed CSF sterility by the end of amphotericin induction therapy.

[b] Medians with 25^{th} percentile to 75^{th} percentile, interquartile range (IQR); unless otherwise noted.

[c] P-values from chi-square test for frequencies and Wilcoxon ranksum test for medians comparing early to late sterility.

[d] CSF parameters from lumbar punctures performed at time of diagnosis.

Those reaching early sterility were more likely to be female and to have a fever, higher baseline CD4 count, lower CSF opening pressure, lower baseline fungal burden (lower CSF quantitative culture and CRAG titer), faster CSF rate of clearance, and higher CSF white cell counts than those not reaching sterility until after the 12^{th} day of therapy (table 4.5 and 4.6). In fact, the later sterility group seemed more similar to the individuals not reaching CSF sterility during induction therapy than to the early sterility group.

Table 4.6: Univariate logistic regression associations with early sterility versus later sterility during amphotericin therapy among individuals with cryptococcal meningitis in the COAT trial.

Covariate	N	OR (95% CI) [a]	P-value
Males (versus females)	81	0.31 (0.12, 0.77)	0.01
Axillary temperature (^oC)	81	1.56 (1.00, 2.42)	0.05
Fever (axillary temperature >37.5^oC)	81	4.31 (1.48, 12.56)	0.01
Serum potassium (mmol/L)	70	2.02 (0.86, 4.73)	0.11
CD4 count (cells/μL)	81	1.02 (1.01, 1.03)	0.004
Opening pressure (cmH_2O)	64	0.96 (0.92, 1.00)	0.04
CSF quantitative cryptococcal culture ($\log_{10}$ CFU/mL)	76	0.53 (0.37, 0.78)	0.001
CSF white blood cells (/10 μL)	77	1.04 (1.01, 1.07)	0.03
CSF cryptococcal antigen titer ($\log_2$ 1:titer)	78	0.77 (0.63, 0.94)	0.01
Early fungicidal activity, over 1 week (x10 $\log_{10}$ CFU/mL/day)	71	1.45 (1.13, 1.87)	0.004

[a] Odds ratio (OR) and 95% confidence interval (CI)

4.4 Conclusions and Discussion

Cryptococcal meningitis (CM) is an under-recognized opportunistic infection claiming the lives of nearly 500,000 individuals every year [23]. Treatment for CM has vastly improved in many tertiary care settings throughout the world with the use of amphotericin B, and currently treatment guidelines suggest an amphotericin-based treatment regimen for two weeks for the induction, or initial, phase of therapy. However, with these recommendations, many CM patients will continue to have viable *C. neoformans* in their CSF. This analysis assessed the frequency of and predictive factors for CSF sterility among HIV-positive, ART-naïve, individuals from Uganda or South Africa experiencing a first episode of CM.

Among all individuals randomized to the Cryptococcal Optimal ART Timing (COAT) trial, 46% achieved CSF culture sterility by the end of two weeks of induction therapy with

amphotoericin and fluconazole. The frequency of sterility was similar to that observed in other settings where initial antifungal therapy consisted of amphotericin either alone or in combination with fluconazole: 48% in a contemporary South African setting with two weeks of amphotericin alone [43]; 44% in Uganda with two weeks of amphotericin alone [30]; 45% with amphotericin alone in Brazil [78]; 37% with amphotericin alone and 40% with amphotericin plus fluconazole in France [40]; 51% with amphotericin alone in a US-based clinical trial [25]; and 52% with amphotericin alone and 63% with amphotericin plus fluconazole over 6-months of follow-up in Thailand [85]. Higher rates of sterility have been observed when amphotericin is combined with flucytosine for induction therapy (54-74%) [40, 41, 85]; however, flucytosine is not commonly available in sub-Saharan Africa outside of research protocols.

While amphotericin has potent antifungal activity, it is well recognized to be fraught with toxicities such as infusion-related side effects (fever, chills, rigors), electrolyte abnormalities, and nephrotoxicity, including acute kidney injury [79, 80, 86]. Side effects can be minor, but can also lead to treatment discontinuations, irreversible reductions in kidney function, increased hospital stays, and death [80, 86]. Different therapeutic strategies, most notably customized or patient-specific CM treatment, may help to limit side effects while maintaining treatment success. For example, shortened therapy could be given to patients with good prognosis for sterility. Conversely, patients with clinical characteristics suggestive of not reaching CSF sterility could be given more potent therapy. The efficacy of such a customized approach to cryptococcal induction therapy would require a randomized controlled trial.

What is clear from prior studies, and what this study augments, is that the cryptococcal burden in the CSF at the time of diagnosis is perhaps the clearest predictor of sterility by the end of induction therapy. The CSF fungal burden is indicative of the magnitude of the infection and also reflects the duration of infection. When cryptococcal burden was

measured by CSF quantitative culture in the COAT participants, each $\log_{10}$ increase in the baseline *C. neoformans* CFU/mL was associated with a 51% decline in the odds of sterility, after accounting for baseline hemoglobin and the early fungicidal activity (EFA) over the first week of therapy. A contemporary study from South Africa found a similar association with an unadjusted 30% decline in odds per CFU/mL increase in baseline fungal burden [43] and a retrospective evaluation of CM patients in Brazil suggested a 94% lower odds of sterility for patients with microscopy-based quantitative counts of at least 10 yeast cells/μL of CSF [78]. Findings based on CSF CRAG titers told a similar story in the COAT trial with each doubling of the titer associated with a 17% decline in odds of sterility, after adjustment. High CSF CRAG titers at diagnosis, either greater than 1:512 or greater than 1:1024, have previously been associated with greater odds of non-sterility in cohorts from France and the US [40, 41]. In the current study population, CSF CRAG titers greater than 1:512 were associated with 3.5-fold higher odds of non-sterility (95% CI: 1.1, 11.0) and titers greater than 1:1024 were associated with a 3.4-fold higher odds of non-sterility (95% CI: 1.3, 8.5).

Baseline hemoglobin was also found to be a significant, independent risk factor for CSF sterility. Other studies have not described a relationship between baseline hemoglobin and the odds of treatment success during CM induction therapy; though one study did describe an association with serum albumin [41]. Hemoglobin exhibited a small but significant correlation with baseline cryptococcal burden (Pearson correlation coefficient = 0.18, p-value = 0.02) and no evidence of correlation with 1-week EFA (correlation coefficient = 0.13, p-vlaue = 0.11). The correlation with cryptococcal burden may have contributed to the association between hemoglobin and the odds of sterility, as there was only a marginal association between hemoglobin and sterility univariately. Furthermore, the difference in hemoglobin between sterility groups, conferring a 19% reduced odds of sterility with each g/dL increase, is likely not as clinically meaningful of a marker as the quantity of

cryptococcus in the CSF.

The calculated rate of clearance was also found to be significantly associated with the odds of reaching CSF sterility in this patient population; however, the practical utility of this association is questionable as these data may not be accessible when the probability of treatment success and decisions about treatment regimen are being considered. The EFA during the first week depends on knowing culture results at baseline and, at the very least, around the end of the first week of amphotericin. However, cultures may take up to 14 days of incubation prior to growth, particularly when burdens are low. Consequently, the EFA may not be a practical factor to use for prediction and customizing therapy.

With the caveats of EFA and hemoglobin, the relationship between sterility and baseline fungal burden may be more suitable for generating risk profiles, as it is biologically sound, has been confimed both in and outside of highly-resourced countries, and resources for conducting either CSF fungal cultures or CSF CRAG testing are available in many settings. Another beneficial characteristic of this relationship is that it appears not to be confounded by other baseline demographics or clinical characteristics.

In light of these advantages, this analysis investigated binary thresholds of fungal burden for use in discriminating patients who did and did not have favorable probability of reaching CSF sterility by the end of amphotericin therapy. For fungal burdens based on CSF culture, a cutpoint of 10,000 CFU/mL had a favorable trade-off between high specificity and increased sensitivity. The vast majority, of individuals with cultures 10,000 CFU/mL or less had a quantitative culture that was sterile, or low, when assessed between day 5 and 11 of induction therapy. Furthermore, the unadjusted predicted probability of sterility was at least 71% for patients with baseline fungal burdens of 10,000 CFU/mL or less, possibly suggesting that individuals below the cutpoint may not need a full 14 days of amphotericin. However, it is important to note that 75% of the participants in the COAT trial had baseline fungal cultures greater than 10,000 CFU/mL and would still benefit from

the currently recommended 2-week induction strategy.

As CRAG titers may be more simple to measure in practice and may be available before the culture results, a similar analysis was used to identify cut-point of a 1:1024 titer or lower with moderate specificity (76%). Similar to the quantitative culture threshold, a large majority of individuals below the CRAG cut-point had a mid-treatment quantitative culture of 100 CFU/mL or less and the crude predicted probability of sterility was at least 65% in this group. One limitation to the generalizability of this CRAG titer threshold is that, while different methods for estimating CRAG titer are strongly correlated, the absolute values of the titers can differ. The difference between CRAG titers from a latex agglutination assay compared to the lateral flow assay was recently described, and it was found that LFA titers were roughly 2.5 dilutions higher than titers estimated in the same sample using latex agglutination [87]. The current analysis was only able to assess CRAG titer cut-points based on estimates using the LFA, thus settings using a latex agglutination platform may find a lower cut-point to be more suitable.

As mentioned above, the field of CM care and treatment is currently focused on finding treatment strategies that make use of the potency of amphotericin's fungicidal activity while mitigating its toxicity and making therapy more suitable to less-resourced areas. One particular strategy includes shortening the duration of amphotericin administration to 5 or 7 days [57, 59]. The cut-point analysis highlights how data on the degree of infection could be used in practice to inform treatment choices. Synthesis of the literature and this analysis suggest that patients with lower baseline fungal burdens (perhaps $\leq$ 10,000 CFU/mL or $\leq$ 1:1024 titers) have the greatest probability of treatment success with short-course amphotericin and patients with higher burdens have much greater probability of having residual infection if amphotericin is shortened. This residual burden is not without consequence, namely resulting in higher rates of CM relapse [43, 83], neurologic deterioration [43], and potentially fatal cryptococcal-related immune reconstitution inflammatory

syndrome (IRIS) [43, 58]. Therefore, the current data support the conclusion that shortening CM induction therapy may best be applied in a customized manner, considering baseline fungal burden.

It should be noted that all patients enrolled in the COAT trial received 14 days of amphotericin plus fluconazole induction therapy, and it can only be inferred whether patients may benefit from different treatment durations. It was not possible, with these data, to assess whether patients with lower levels of infection would have had successful outcomes with a shorter course of amphotericin, and it will be important to assess the effects of shortened or augmented interventions in dedicated studies. Furthermore, the positive predictive value of the cut-points of 10,000 CFU/mL and 1:1024 CRAG titer were 86% and 70%, respectively, suggesting that many more patients with burdens of infections greater than the cut-points did have sterile CSF cultures by the end of treatment.

A limitation of the current analysis is possible misclassification of the sterility outcome, as sterility may have occurred shortly after the end of induction therapy. One benefit of amphotericin is that it is known to have a long half-life, up to 15 days for terminal elimination [88], and its antifungal activity will likely continue after administration has stopped. Furthermore, treatment guidelines suggest continuation of fluconazole, called consolidation therapy, in doses of 400-800 mg/day after the initial amphotericin regimen. The combined activity of residual amphotericin and consolidation therapy will likely result in eventual CSF sterility for many patients in whom sterility is not observed by the end of induction therapy. As such, a limitation of this analysis is that the residual antifungal effects of amphotericin and continuing fluconazole therapy were not considered in defining sterility. Half of the individuals surviving beyond induction therapy with non-sterile CSF cultures in the COAT trial had final fungal burdens lower than 100 CFU/mL and may have experienced CSF sterility soon after induction therapy ended. When these subjects would have reached sterility after induction therapy ended can be inferred using estimates of the

EFA for the consolidation regimen. Participants in the COAT trial received 800 mg/day fluconazole for 3 weeks following induction therapy. Based on estimates of the EFA with 800 mg fluconazole per day for induction therapy (0.07 $\log_{10}$ decline in CFU/mL/day [89]), those with a final culture less than 100 CFU/mL may have reached CSF sterility no later than 29 days after induction therapy ended. This rate may be an underestimate of the clearance because, as mentioned, there is likely some residual activity of amphotericin while the drug is being cleared from the body. Exploratory analysis was conducted combining individuals with fungal burdens less than 100 CFU/mL with individuals who reached CSF sterility (Appendix B, section B.2). Similar multivariable associations were seen.

In conclusion, this analysis of CM patients randomized to the COAT trial found that 46% of HIV-positive individuals in Uganda and South Africa had sterile CSF after two weeks of amphotericin and fluconazole (800mg/day) combination induction therapy. Sterility by the end of induction therapy, and sterility before the 12^{th} day of induction, was highly related to lower baseline cryptococcal burdens, lower baseline hemoglobin, and faster rates of cryptococcal clearance in the first week of therapy. Patients with cryptococcal burdens below 10,000 CFU/mL may not need a full two weeks of amphotericin therapy to completely sterilize the CSF as sterility was observed in the majority of these patients prior to the end of therapy. However, two weeks of potent induction therapy seemed to be needed for the average patient who presents with high fungal burden.

4.5 Exploratory Analysis

4.5.1 CSF Cytokines

Exploratory analysis was conducted on possible immunological markers of CSF sterility after amphotericin therapy. Numerous cytokines and chemokines were measured in CSF

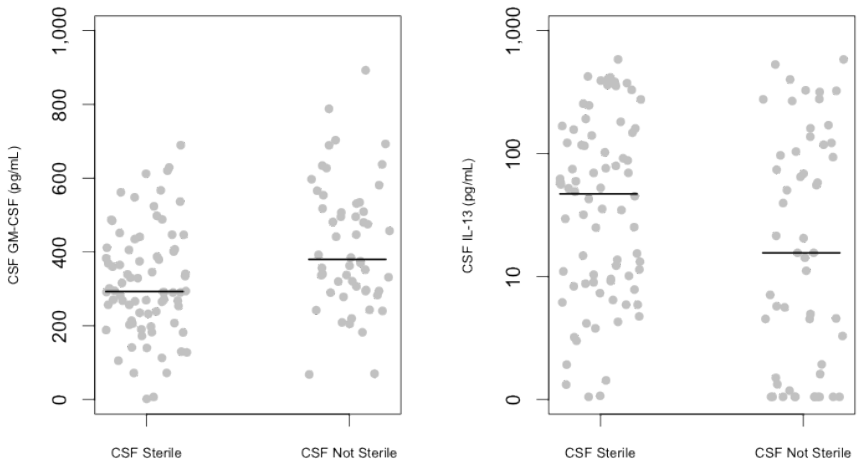

Figure 4.5: CSF cytokine levels by CSF culture status by the end of two weeks of amphotericin therapy among individuals with cryptococcal meningitis in the COAT trial.

samples collected at diagnosis, which were subsequently cryopreserved (-80^{o}C) and transported to the University of Minnesota for testing. The following cytokines were measured in a multiplex Luminex platform (in pg/mL; Bio-Rad, Hercules, CA): interleukin (IL)-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17, granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein 1 alpha and 1 beta (MIP-1α, MIP-1β), and vascular endothelial growth factor (VEGF). All cytokine levels were $\log_2$ transformed for analysis. MIP-1α and VEGF were later added to the assay and, therefore, are not measured in all participants. Levels of IL-1β, IL-2, and IL-5 were below the level of detection in a sizeable number of individuals, thus these cytokines were dichotomized into detectable or undetectable for analysis.

Table 4.7: Distribution of CSF cytokines among individuals with cryptococcal meningitis in the COAT trial with sterile CSF, versus subjects with non-sterile CSF, by the end of amphotericin therapy.

	N	Sterile at 2 weeks	Not sterile at 2 weeks	OR (95% CI) [a]	P-value
N per group		78 (54%)	54 (37%)		
N undetectable (%) [b]					
IL-1β	132	31 (40%)	31 (57%)	0.49 (0.24, 0.99)	0.05
IL-2	132	32 (41%)	23 (43%)	0.94 (0.46, 1.89)	0.86
IL-5	132	29 (37%)	25 (46%)	0.69 (0.34, 1.39)	0.30
Median [25th, 75th percentile] ($\log_2$ pg/mL)					
IL-4	132	-0.6 [-2.0, 0.4]	-0.1 [-1.0, 0.7]	0.89 (0.70, 1.13)	0.34
IL-6	132	7.8 [5.6, 9.8]	6.7 [4.6, 8.5]	1.10 (0.97, 1.24)	0.14
IL-7	132	2.3 [1.7, 3.3]	1.9 [1.0, 3.2]	1.11 (0.93, 1.33)	0.25
IL-8	132	9.6 [8.3, 10.6]	9.1 [8.5, 10.2]	1.10 (0.88, 1.36)	0.40
IL-10	132	3.0 [2.1, 4.1]	3.2 [2.3, 3.7]	1.00 (0.77, 1.29)	>0.99
IL-12	132	3.5 [2.6, 4.2]	3.3 [1.7, 4.1]	1.10 (0.89, 1.36)	0.36
IL-13	132	5.6 [3.2, 7.1]	4.0 [0.6, 6.9]	1.15 (1.01, 1.31)	0.03
IL-17	132	4.0 [2.6, 5.0]	4.0 [1.8, 5.0]	0.97 (0.84, 1.13)	0.71
G-CSF	132	6.3 [5.4, 7.2]	6.0 [5.1, 7.2]	1.05 (0.84, 1.30)	0.67
GM-CSF	132	8.2 [7.7, 8.7]	8.6 [8.2, 9.1]	0.46 (0.27, 0.80)	0.006
IFN-γ	132	5.4 [3.9, 6.8]	5.5 [4.4, 6.9]	0.99 (0.85, 1.16)	0.91
TNF-α	132	3.6 [2.7, 5.8]	3.2 [2.5, 5.1]	1.12 (0.95, 1.33)	0.19
MCP-1	132	8.7 [7.7, 10.0]	9.1 [7.3, 10.9]	0.94 (0.79, 1.12)	0.47
MIP-1α	64	4.1 [2.1, 6.5]	4.4 [3.0, 6.9]	0.89 (0.71, 1.10)	0.28
MIP-1β	132	6.4 [5.4, 7.2]	6.6 [5.3, 7.2]	1.00 (0.82, 1.22)	0.97
VEGF	89	5.4 [2.8, 6.6]	5.6 [3.5, 6.6]	0.94 (0.79, 1.12)	0.48

[a] Univariate odds ratios (OR) and 95% confidence intervals (CI) from logistic regression comparing odds of sterility to the odds of non-sterility.

[b] Cytokine levels for IL-1β, IL-2, and IL-5 were dichotomized into detectable and undetectable. The proportion of subjects with undetectable levels is presented and the odds of sterility in those with undetectable versus detectable levels was modeled.

CSF samples were available for 132 (96%) of the 138 individuals who reached either sterility or non-sterility. Few CSF cytokines were found to be associated with sterility (table 4.7). However, individuals with a sterile CSF by the end of two weeks of amphotericin had significantly lower levels of GM-CSF, slightly higher levels of IL-13 and were less likely to have undetectable IL-1β. At an alpha threshold of 0.01, to partially account for finding spurious results due to multiple comparisons, only GM-CSF remained statistically significant. The differences in GM-CSF and IL-13 between the sterility groups were not particularly striking when explored graphically and may not reflect clinically significant differences (figure 4.5).

When GM-CSF was investigated in multivariable analysis with baseline fungal burden, 1-week EFA, and baseline hemoglobin, a 1-unit change in GM-CSF on the $\log_2$ scale - or a doubling in GM-CSF - was associated with 70% lower odds of reaching sterility at the end of amphotericin therapy (OR = 0.30, 95% CI: 0.14, 0.66); though the range of observed GM-CSF concentrations was limited in this cohort and a doubling in GM-CSF concentration would represent a comparison of more extreme values. Detectable IL-1β was associated with 79% lower odds of CSF sterility at the end of amphotericin, after accounting for baseline quantitative culture, 1-week EFA, and baseline hemoglobin (OR = 0.21, 95% CI: 0.61, 0.95). IL-13 was found to be marginally independently associated with sterility after accounting for baseline fungal burden, 1-week EFA, and baseline hemoglobin (OR = 1.20, 95% CI: 1.01, 1.41), but the association diminished after additionally accounting for GM-CSF and detectable IL-1β (OR for IL-13 = 1.00, 95% CI: 0.81, 1.22).

The results of the final adjusted model, including baseline quantitative culture, hemoglobin, 1-week EFA, GM-CSF and IL-1β, are presented in table 4.8.

GM-CSF is a cytokine – secreted by macrophages, T-cells, and natural killer cells – that is a potent growth signal for white blood cells and a simulant for phagocytosis. In the context of cryptococcal infection, GM-CSF supports successful phagocytosis and

Table 4.8: Multivariable logistic regression of characteristics related to sterile versus non-sterile CSF by the end of amphotericin therapy among individuals with cryptococcal meningitis in the COAT trial

	Model Estimates
Observations in the model	110
Number reaching sterility (%)	69 (63%)
Number not reaching sterility (%)	41 (37%)
Model C statistic [a]	0.863
	OR (95%CI) [b]
CSF quantitative cryptococcal culture ($\log_{10}$ CFU/mL)	0.45 (0.28, 0.71)
Early fungicidal activity, day 0 to 11 (x10 $\log_{10}$ CFU/mLday)	1.52 (1.18, 1.95)
Hemoglobin (g/dL)	0.78 (0.62, 0.98)
GM-CSF ($\log_2$ pg/mL)	0.37 (0.17, 0.80)
Undetectable IL-1β (vs. detectable)	0.27 (0.09, 0.79)

[a] Model C statistic is a measure of model prediction describing the probability that the model-estimated odds of the outcome, among those with the outcome, is greater than the model-estimated odds in those without the outcome. This is equivalent to the area-under-the-curve. Values close to 0.5 indicate model prediction no better than chance, whereas values above 0.8 suggest models with strong prediction.

[b] Odds ratio (OR) and 95% confidence interval (CI)

intracellular killing of *C. neoformans in vitro* [1, 90–92] and has been shown to enhance the fungicidal activity of voriconazole [93] and fluconazole [94] *in vitro*. Perhaps counter-intuitively, in this study lower levels of CSF GM-CSF were seen in individuals who reached CSF sterility compared to those who did not reach sterility. Few studies have looked at GM-CSF levels in the CSF at the time of CM diagnosis [95], thus the relative understanding of GM-CSF and its interaction with *C. neoformans* may not be complete. IL-1β is a pro-inflammatory cytokine produced by activated macrophages, thus the increased detection of IL-1β among those who reached CSF sterlity by the end of amphotericin therapy in the current study may be an indication of greater macrophage activity in individuals who reach sterility compared to those who did not. Additionally, many cytokines and chemokines were tested in this analysis, and it is possible that the observed association with GM-CSF and

IL-1β may have been spurious.

As the EFA is strongly related to the probability of successful CSF sterility at the end of induction therapy and IFN-γ has been associated with EFA [58, 95], it was hypothesized that this analysis would also identify IFN-γ as an important biomarker of sterility. However, this was not found. Concentrations of IFN-γ in the CSF at the time of CM diagnosis were not associated with either the odds of CSF sterility nor correlated with the 1-week or 2-week EFA (Pearson correlation coefficient = -0.05, p-value = 0.59 and correlation coefficient = -0.01, p-value = 0.93, respectively).

4.5.2 Treatment Failure

An alternative approach to address the objective of this chapter would be to assess differences between treatment success and treatment failure, where failure is defined as either death or a non-sterile CSF culture at the end of induction therapy. The primary focus of this chapter considered individuals with non-sterile CSF as seperate from individuals who died during induction therapy because of the potential heterogeneity of these groups. However, exploratory analysis was conducted to assess whether considering death and non-sterile outcomes collectively identified any further characteristics that differentiate from individuals with treatment success, or sterile cultures by the end of induction therapy.

Univariate logistic regression identified very similar baseline characteristics that differentiate treatment success from failure and CSF sterility from non-sterility; namely, axillary temperature, CD4 count, CSF white blood cell count, CSF fungal burden (by quantitative culture and cryptococcal antigen titer), and the rate of clearance over the course of treatment (table 4.9). Baseline hemoglobin was not found to be associated with treatment success versus treatment failure.

Stepwise multivariable regression analysis showed that CSF quantitative culture and the rate of clearance over the first week of therapy were highly predictive of treatment

Table 4.9: Univariate logistic regression associations with treatment success versus treatment failure by the end of amphotericin therapy among individuals with cryptococcal meningits in the COAT trial

Covariate	N	OR (95% CI) [a]	P-value
Age (years)	154	0.97 (0.93, 1.00)	0.08
Males (versus females)	154	0.62 (0.33, 1.17)	0.14
Axillary temperature (^oC)	152	1.79 (1.25, 2.55)	0.001
Fever (axillary temperature >37.5^oC)	152	3.57 (1.45, 9.09)	0.006
Hemoglobin (g/dL)	150	0.91 (0.80, 1.03)	0.14
Hematocrit (%)	150	0.97 (0.93, 1.01)	0.18
CD4 count (cells/μL)	152	1.01 (1.00, 1.01)	0.03
CSF opening pressure (mmH_2O)	132	0.98 (0.95, 1.00)	0.10
CSF opening pressure $\geq$ 250 mmH_2O	132	0.70 (0.35, 1.41)	0.32
CSF white blood cells (/10 μL)	145	1.03 (1.00, 1.05)	0.04
CSF quantitative cryptococcal culture ($\log_{10}$ CFU/mL)	145	0.56 (0.42, 0.76)	<0.001
CSF cryptococcal antigen titer ($\log_2$ 1:titer)	146	0.86 (0.75, 0.98)	0.02
Early fungicidal activity, over 1 week (x10 $\log_{10}$ CFU/mL/day)	128	1.32 (1.11, 1.56)	0.002
Early fungicidal activity, over 2 weeks (x10 $\log_{10}$ CFU/mL/day)	149	2.86 (1.93, 4.24)	<0.001

[a] Odds ratio (OR) and 95% confidence interval (CI)

success, compared to treatment failure (table 4.10). The regression also indicated that axillary body temperature was additionally related to treatment success, versus failure.

Table 4.10: Multivariable logistic regression of characteristics related to treatment success versus treatment failure by the end of amphotericin therapy among individuals with cryptococcal meningits in the COAT trial.

	Full Model	Reduced Model 1	Reduced Model 2	Reduced Model 3	Reduced Model 4
Observations in the model	101	117	119	125	126
Number with treatment success (%)	53 (52%)	67 (57%)	67 (56%)	70 (56%)	70 (56%)
Number with treatment failure (%)	48 (48%)	50 (43%)	52 (44%)	55 (44%)	56 (44%)
Model C statistic [a]	0.806	0.802	0.801	0.808	0.788
	OR (95%CI) [b]	OR (95%CI)	OR (95%CI)	OR (95%CI)	OR (95%CI)
CSF quantitative cryptococcal culture ($\log_{10}$ CFU/mL)	0.49 (0.30, 0.79)	0.50 (0.32, 0.76)	0.49 (0.33, 0.73)	0.48 (0.33, 0.70)	0.46 (0.31, 0.67)
Early fungicidal activity, day 0 to 11 (x10 $\log_{10}$ CFU/mLday)	1.30 (1.06, 1.60)	1.34 (1.09, 1.65)	1.35 (1.10, 1.67)	1.38 (1.12, 1.71)	1.42 (1.16, 1.73)
Axillary temperature (^{o}C)	1.45 (0.91, 2.33)	1.57 (1.00, 2.47)	1.58 (1.01, 2.49)	1.61 (1.03, 2.53)	
CSF white blood cells (/10 μL)	1.01 (0.98, 1.03)	1.00 (0.98, 1.02)	1.00 (0.99, 1.02)		
CD4 count (cells/μL)	1.00 (0.99, 1.01)	1.00 (0.99, 1.01)			
CSF opening pressure $\geq$250 mmH_2O	1.03 (0.36, 2.94)				

[a] Model C statistic is a measure of model prediction describing the probability that the model-estimated odds of the outcome, among those with the outcome, is greater than the model-estimated odds in those without the outcome. This is equivalent to the area-under-the-curve. Values close to 0.5 indicate model prediction no better than chance, whereas values above 0.8 suggest models with strong prediction.

[b] Odds ratio (OR) and 95% confidence interval (CI)

4.5.3 Baseline associations with quantity of residual fungus

Exploratory analysis was conducted to assess, among individuals who did not achieve a sterile CSF by the end of amphotericin induction therapy, characteristics that were predictive of the amount of fungus that was remaining. The quantity of residual fungus was assessed from CSF quantitative culture taken at the end of amphotericin therapy. Fifty-seven individuals had a non-sterile CSF and had a quantitative fungal culture available to be included in this exploratory analysis.

Table 4.11: Univariate linear associations with quantity of residual fungal infection at the end of amphotericin-based induction therapy among individuals with cryptococcal meningitis in the COAT trial.

	N with data	**Regression coefficient (95% CI [a])**	**P-value [a]**
Demographic and clinical characteristics			
Age (years)	57	0.01 (-0.03, 0.04)	0.75
Males, N (%)	57	-0.06 (-0.70, 0.58)	0.86
Glasgow Coma Scale <15	56	-0.07 (-0.80, 0.66)	0.84
Axillary temperature (^{o}C)	56	-0.05 (-0.36, 0.26)	0.76
Fever (axillary temperature >37.5^{o}C)	56	-0.38 (-1.31, 0.55)	0.42
Hemoglobin (g/dL)	56	0.04 (-0.11, 0.19)	0.60
Hematocrit (%)	56	0.02 (-0.04, 0.07)	0.58
CD4 count (cells/μL)	57	-0.002 (-0.01, 0.01)	0.54
HIV viral load ($\log_{10}$ copies/mL)	57	-0.66 (-1.35, 0.02)	0.06
CSF characteristics			
Opening pressure (mmH_2O)	52	-0.01 (-0.03, 0.02)	0.58
Opening pressure > 250 mmH_2O, N (%)	52	-0.29 (-0.97, 0.39)	0.41
Quantitative cryptococcal culture ($\log_{10}$ CFU/mL)	53	0.16 (-0.14, 0.45)	0.30
Cryptococcal antigen titer (1:x)	55	0.02 (-0.10, 0.15)	0.71

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Table 4.11 – continued from previous page

	N with data	Regression coefficient (95% CI [a])	P-value [a]
Cryptococcal antigen titer > 1:2000	55	0.62 (-0.28, 1.53)	0.18
White blood cells (/10 μL)	53	0.01 (-0.03, 0.04)	0.78
Changes in CSF characteristics over time			
Early fungicidal activity, day 0 to 11 ($\log_{10}$ CFU/mL/day)	43	-0.15 (-0.30, -0.002)	0.05
Early fungicidal activity, day 0 to 14 ($\log_{10}$ CFU/mL/day)	57	-0.60 (-0.84, -0.35)	<0.001

[a] Regression coefficient, 95% confidence intervals (CI), and p-values from univariate linear regresion with the quantitative culture burden ($\log_{10}$ CFU/mL) at the end of amphotericin-based induction thearpy as the dependent variable.

Only the early fungicidal activity (EFA) over the full 2 weeks of induction therapy was found to be significantly related to the amount of residual infection (table 4.11). The EFA during the first week and the HIV viral load demonstrated trends towards an association.

Chapter 5

Effect of Cerebrospinal Fluid Sterility at the End of Amphotercin-based Therapy for Cryptococcal Meningitis on Subsequent 3-week and 6-month Mortality

Introduction Cryptococcal meningitis (CM) affects hundreds of thousands of individuals with AIDS every year. Potent amphotericin-based antifungal therapy has reduced mortality rates from CM, however, nearly half of all patients are left with residual fungal infection in their cerebrospinal fluid (CSF). Few contemporary estimates are available on

the consequences of residual infection shortly after the end of amphotericin therapy. The objective of this analysis was to evaluate the association between residual cryptococcal infection and mortality 3 weeks and 6 months following the end of amphotericin therapy in a cohort of HIV-positive individuals with CM in sub-Saharan Africa.

Methods HIV-positive individuals with a first episode of CM, from Uganda and South Africa, enrolled in the Cryptococcal Optimal Antiretroviral (ART) Timing (COAT) trial were eligible for inclusion in this cohort. Patients were treated for CM with 2 weeks of 0.7-1.0 mg/kg/day amphotericin plus 800 mg/day fluconazole induction therapy, followed by consolidation therapy consisting of 3 weeks of 800 mg/day fluconazole then 8 weeks of 400 mg/day fluconazole. Of the 177 individuals enrolled in COAT, 154 survived to the end of amphotericin therapy and were considered for analysis. Individuals were classified as having sterile CSF or non-sterile CSF at the end of induction therapy on the basis of CSF fungal cultures. Cox proportional hazards regression was used to assess the risk of all-cause and CM-related mortality 3 weeks and 6 months after induction therapy among those with and without sterile CSF. Models considered the presence of residual fungus as both a binary indicator as well as in continuous and categorical forms. Univariate and multivariable models, adjusted for COAT treatment arm and baseline fungal burden, were considered.

Results Of the 154 individuals who survived to the end of induction therapy, 75 (49%) had a sterile CSF, 57 (37%) had a non-sterile CSF, and 22 (14%) had no culture at the end of induction therapy to assess sterility status. Forty individuals died within 6 months of the end of induction therapy, of which 23 deaths (58%) occurred within 3 weeks of ending amphotericin. Of those with known sterility status, 13% with a sterile culture and 23% without a sterile culture died by the 3 week endpoint (adjusted HR: 1.5, 95% CI: 0.6,

3.5). There was no evidence of an association between 6-month mortality and sterility (adjusted HR: 1.2, 95% CI: 0.6, 2.3). Deaths attributable to the initial CM episode were more frequent shortly after the end of induction therapy, compared to over 6 months of follow-up. However, there was no evidence that the risk of CM-related death differed by CSF sterility.

Conclusions This analysis did not provide evidence that residual fungal burden is associated with higher all-cause, or CM-related, mortality after amphotericin-based therapy. The results suggest that, with ongoing high-dose consolidation therapy, there does not appear to be a large impact of incomplete clearance of cryptococcus on short-term mortality.

5.1 Introduction

Cryptococcal meningitis (CM), an opportunistic fungal disease occurring in severely immunosuppressed individuals, accounts for upwards of 20-44% of AIDS-associated mortality in sub-Saharan Africa [96–99]. With potent amphotericin-based antifungal therapy, 10-week mortality from CM can be as low as 12-20% [29–31]. This lower mortality rate is seen despite the fact that 50-70% of HIV-positive individuals will continue to have detectable *Cryptococcus neoformans* in their cerebrospinal fluid (CSF) at the end of two weeks of amphotericin treatment [25, 40, 41]. A consolidation regimen of additional antifungal therapy with fluconazole, after amphotericin induction therapy, is recommended to further suppress the infection and support long-term recovery and may contribute to the lower overall mortality rates.

Some studies have suggested an association between incomplete sterilization of the CSF after induction therapy and detrimental outcomes, despite consolidation therapy. Two cohort studies and a clinical trial of HIV-positive individuals with CM from the 1990's in the

US and Thailand suggested that individuals who had a non-sterile culture at the end of amphotericin-based therapy had higher odds of positive cultures after consolidation therapy [25, 41] and higher odds of 6-month mortality [83], compared to those with a sterile culture. Patients in these studies were treated with up to 400 mg/day fluconazole for consolidation therapy after induction therapy. A more recent study of 106 CM patients starting antiretroviral therapy for HIV in South Africa, treated with amphotericin monotherapy and 400 mg/day fluconazole for consolidation therapy, found a non-significant reduction in mortality 6 months after CM diagnosis among those who had a sterile culture at the time amphotericin therapy was stopped [43]. Aside from the recent South African cohort, no other cohorts have described the role of culture sterility and mortality after an episode of CM in sub-Saharan Africa, which largely bears the burden of CM.

This analysis aims to add to the contemporary literature investigating the impact of sterility at the end of amphotericin on 6-month mortality. Additionally, mortality within the first month after CM diagnosis is quite high and may account for the majority of deaths after therapy is completed. The role of CSF sterility on short-term mortality has not been described previously. Thus, a further objective was to assess whether incomplete sterilization of the CSF after induction therapy contributes to increased mortality 3 weeks later.

5.2 Methods

5.2.1 Study Population and CM Treatment

Subjects included in this analysis were enrolled in the Cryptococcal Optimal Antiretroviral (ART) Timing (COAT) trial, a randomized clinical strategy trial for HIV-positive, ART-naïve individuals with a first episode of CM to determine whether early ART initiation (1-2 weeks after CM diagnosis; prior to hospital discharge) results in superior 26-week

survival compared to deferred ART initiation (5 weeks after diagnosis; as an outpatient). Individuals were eligible for enrollment and randomization in the COAT trial if they were at least 18 years old, receiving amphotericin-based CM treatment, willing to attend regular clinic visits, and provided informed consent. Individuals who were pregnant, breastfeeding, on immunosuppressive therapy, unable to take oral medication, had contraindications to study medications, had been on antifungal therapy for more than one week, or had a prior episode of CM were all excluded. Screening began in November 2010 and ended in April 2012, during which time 177 individuals were randomized from three sites: Mulago Hospital/Infectious Diseases Institute in Kampala, Uganda; Mulago-Mbarara Joint AIDS Program and Mbarara University of Science and Technology in Mbarara, Uganda; and GF Jooste Hospital in Cape Town, South Africa. Approval for the COAT trial was granted by the research ethic committees at the University of Minnesota, Makerere University, Mulago Hospital, Mbarara University, Uganda National Council of Science and Technology, University of Cape Town, South African Medicines Control Council, and National Institutes of Health's National Institute for Allergy and Infectious Diseases Clinical Science Review Committee (www.clinicaltrials.gov: NCT01075152). Approval for this analysis was additionally granted by the Institutional Review Board at the University of Minnesota.

All participants underwent a lumbar puncture for diagnosis of CM and diagnosis was confirmed with either a positive CSF cryptococcal culture or positive CSF cryptococcal antigen (CRAG) latex agglutination assay. Induction therapy consisted of 0.7-1.0 mg/kg amphotericin B daily in combination with 800 mg fluconazole daily for two weeks. All individuals also received intravenous fluids and electrolyte supplementation during amphotericin therapy. Therapeutic lumbar punctures, to control intracranial pressure, were conducted according to treatment guidelines [37]; in addition, the COAT protocol specified additional lumbar punctures to be done after 7 and 14 days of amphotericin to monitor clearance of *C. neoformans*. Regardless of sterility at the end of amphotericin therapy,

all individuals received 3 further weeks of 800 mg/day fluconazole followed by 8 weeks of 400 mg/day fluconazole and at least one year of 200 mg/day fluconazole. If patients were found to have a positive culture at the end of amphotericin therapy, the study protocol recommended that 800 mg/day fluconazole be continued until the CSF was sterile, at which time the dosage could be reduced to 400 mg/day.

Randomization of eligible individuals occurred after 7 to 11 days of amphotericin therapy. Those randomized to the early arm initiated ART therapy within 48 hours of randomization, while those randomized to the deferred arm initiated ART 4 weeks after randomization. All individuals initiated combination ART with either efavirenz plus zidovudine and lamivudine or efavirenz plus stavudine and lamivudine.

Of 177 individuals, 154 (87%) survived to the end of amphotericin therapy and were considered in this analysis.

5.2.2 CSF Fungal Burden and Definitions of CSF Sterility

All CSF samples obtained from lumbar punctures during amphotericin therapy, including the lumbar puncture conducted at the time of CM diagnosis, were evaluated by qualitative and quantitative fungal culture. Cultures were conducted in microbiology labs, approved by the US National Institutes of Health, at each study site. Briefly, CSF was plated onto Sabouraud's dextrose agar and incubated at 30^oC to allow fungal growth. Cultures were qualitatively considered positive when *C. neoformans* was detected or negative in the absence of fungal growth after 14 days of incubation. Quantification of *C. neoformans* fungal burden was conducted using 10-fold serial dilutions of CSF, up to $1{:}10^5$ dilution, and counting colony-forming units (CFUs) of *C. neoformans* seen at the most diluted plate with growth [29]. Quantitative culture counts were $\log_{10}$ transformed for analysis.

Sterility of the CSF from *C. neoformans* was determined based on all observed culture results during induction therapy. Individuals were considered to have reached CSF sterility

when a CSF culture was negative and no subsequent positive cultures were found for the remainder of amphotericin induction therapy. Ten individuals were found to have a negative culture followed by a positive culture later during amphotericin therapy. The initial negative cultures for these individuals were considered to be false negatives. Such culture reversions accounted for 0.7% of all observed cultures.

Non-sterility was defined for individuals in whom no negative cultures were observed during amphotericin therapy but for whom a culture was conducted between the 12^{th} day and the end of therapy.

Twenty-two individuals (14% of 154) did not have any negative cultures during the first 12 days of therapy and also did not have a culture after day 12, despite surviving. For these individuals, the sterility outcome was unobserved. The primary analysis was restricted to the 132 individuals with observed outcomes, excluding these 22 individuals. Sensitivity analyses were conducted using multiple imputation to assess the effect of these exclusions on the final model results.

Semi-quantitative CSF cryptococcal antigen (CRAG) titers were estimated using the lateral flow assay (Immy, Inc., Norman, OK).

The rate of cryptococcal clearance from the CSF, also termed the early fungicidal activity or EFA [29], was calculated from the first observed quantitative culture to either the first negative culture or the culture taken at the end of amphotericin therapy, whichever occurred first. Subject-specific linear regression analysis was conducted using $\log_{10}$ CFU/mL as the dependent variable and days of amphotericin as the independent variable (see Appendix B, section B.1). The EFA is estimated by the slope of the regression equation for a particular individual. The EFA times -1 was used in analysis, such that a higher positive value denoted a faster rate of clearance.

5.2.3 Cause of Death

All deaths had a primary cause of death initially determined by the COAT medical officers attending to the patient. All deaths were then, retrospectively, adjudicated by a panel of three independent clinicians with expertise in HIV and cryptococcal meningitis for agreement or disagreement with the cause of death. A concensus of 2 of 3 adjudicators was needed for agreement or disagreement. There was 88% agreement between the site medical officers and the adjudication committee for CM-related deaths, and only deaths that were initially listed as related to initial CM and had agreement by the committee were included in the analysis of CM-related deaths.

5.2.4 Statistical Analysis

Baseline clinical and demographic characteristics, as well as characteristics over the induction phase of treatment, were compared by sterility status. Median values were compared using Wilcoxon ranksum tests and categorical characteristics were compared using χ^2 tests.

Two endpoints were evaluated in this analysis, 1) mortality within 3 weeks of the end of amphotericin and 2) mortality within 6 months of the end of amphotericin therapy. Analyses were conducted for both all-cause mortality as well as for deaths deemed related to initial CM. Cox proportional hazards models were used to assess the association of CSF sterility with the endpoints. Crude models were evaluated, as were models adjusted for COAT treatment arm and additionally adjusted for factors found to be significantly associated with both sterility and mortality. Tests confirmed that the proportional hazards assumption was suitable for all models evaluated (see Appendix C, section C.1).

Sterility was assessed in three different ways for all crude and adjusted models, 1) as a binary indicator (sterile versus non-sterile), 2) a binary indicator plus a continuous variable for the amount of residual cryptococcus found in the final CSF culture (in $\log_{10}$

CFU/mL), and 3) as a categorical variable (sterile, 0-99 CFU/mL, 100-999 CFU/mL, ≥1,000 CFU/mL).

5.2.5 Multiple Imputation

Multiple imputation was used to estimate the primary effect of sterility on mortality after accounting for missing baseline characteristics and sterility after amphotericin therapy. The multiple imputation model consisted of data on age, sex, body weight, estimated rate of cryptococcal clearance, quantitative cultures throughout amphotericin therapy, CSF opening pressures and white cell counts during amphotericin therapy, the days of amphotericin therapy at the final culture, indicator for a sterile culture seen during therapy, COAT treatment arm, and vital status 14 days after the start of amphotericin. The model was used to impute 40 complete datasets. Each dataset was used to estimate the weighted average crude and adjusted hazard ratio of 3-week and 6-month mortality.

Based on this imputation model, 23 of the 40 (56%) imputed datasets had 75 individuals with a sterile CSF culture at the end of amphotericin therapy. As this is the same number as seen in the raw dataset, the imputation model tended to place those with an unknown sterility status in the non-sterile group. Sixteen (40%) imputation datasets had 76 individuals and 1 (3%) imputation dataset had 77 individuals in the sterile group.

5.3 Results

5.3.1 Study Population

One hundred thirty-two individuals survived to the end of amphotericin-based induction therapy and had obesrved cultures available for analysis (figure 5.1). These patients with CM and HIV had similar demographics to other cohorts of CM patients in sub-Saharan

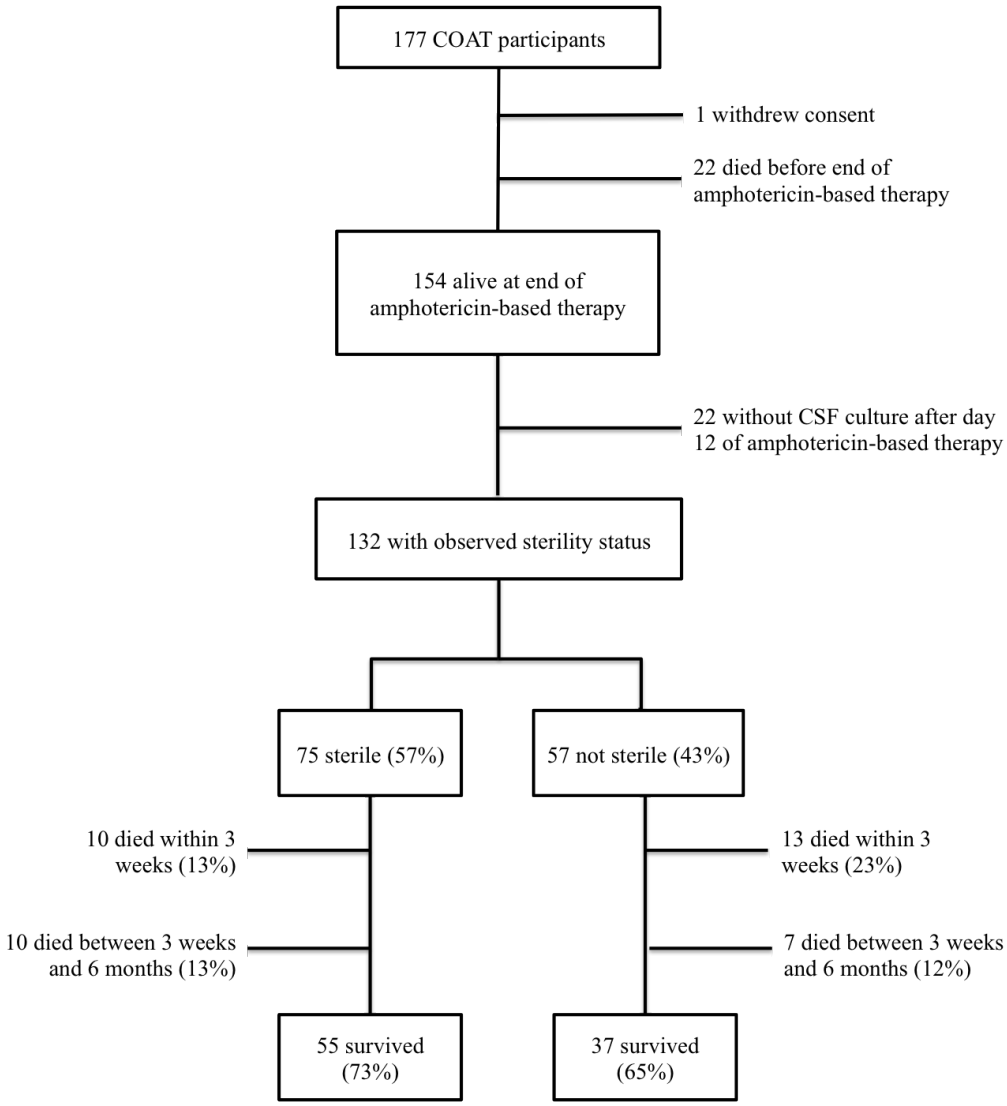

Figure 5.1: Sterility and vital status outcomes for individuals with cryptococcal meningitis in the COAT trial.

Africa [30, 35, 43, 60], namely median age in the mid-30s, roughly half males, a large proportion of patients with altered mental status (Glasgow Coma Score (GCS) less than 15), increased intracranial pressure, a large fungal load in the CSF, and very low CD4 counts.

Table 5.1: Baseline characteristics and outcomes by sterility status at the end of amphotericin therapy for individuals with cryptococcal meningitis in the COAT trial.

	Sterile at end of amphotericin		**Not sterile at end of amphotericin**		
N per group	75 (57%)		57 (43%)		
	N with data	Median (IQR) [a]	N with data	Median (IQR) [a]	P-value [b]
Study site, N (%) [c]	75		57		0.40
Kampala		43 (46%)		39 (42%)	
Mbarara		16 (49%)		10 (30%)	
Cape Town		16 (59%)		8 (30%)	
Treatment Group, N (%) [d]	75		57		0.53
Early ART		37 (49%)		25 (44%)	
Deferred ART		38 (51%)		32 (56%)	
Demographic and Clinical Parameters					
Age (years)	75	34 [27, 40]	57	37 [30, 42]	0.11
Males, N (%)	75	38 (51%)	57	38 (67%)	0.07
Weight (kg)	52	54.0 [46.0, 59.5]	42	54.5 [47.0, 62.0]	0.55
Weight missing, N (%)	75	23 (31%)	57	15 (26%)	0.59
Headache duration (days)	71	14 [7, 28]	56	14 [7, 21]	0.67
Karnofsky Score	75	50 [40, 60]	56	50 [40, 50]	0.97
Glasgow Coma Scale, N (%)	75		56		0.44
<15		22 (29%)		13 (23%)	
15		53 (71%)		43 (77%)	
Heart rate (beats per minute)	75	80 [70, 90]	56	76 [64, 90]	0.21
Respiratory rate (breaths per minute)	74	22 [20, 24]	55	20 [19, 24]	0.20

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Table 5.1 – continued from previous page

	Sterile at end of amphotericin		Not sterile at end of amphotericin		
	N with data	Median (IQR) [a]	N with data	Median (IQR) [a]	P-value [b]
Systolic blood pressure (mmHg)	73	110 [108, 122]	56	110 [106, 120]	0.38
Diastolic blood pressure (mmHg)	73	70 [60, 82]	56	70 [61, 82]	0.61
Axillary temperature (^oC)	75	36.8 [36.2, 37.7]	56	36.3 [35.8, 36.8]	0.01
Fever (axillary temperature > 37.5^oC), N (%)	75	19 (25%)	56	7 (13%)	0.18
Clinical Laboratory Values					
Hemoglobin (g/dL)	73	10.6 [8.9, 12.9]	56	11.7 [10.1, 13.2]	0.06
Hematocrit (%)	73	31.4 [26.7, 37.7]	56	34.6 [29.6, 38.9]	0.11
White blood cells (x$10^3/\mu$L)	73	3.5 [2.5, 5.3]	56	3.4 [2.5, 5.1]	0.96
Creatinine (mg/dL)	73	0.7 [0.5, 0.9]	55	0.6 [0.5, 0.9]	0.61
Potassium (mmol/L)	65	3.9 [3.4, 4.2]	51	4.0 [3.4, 4.3]	0.83
HIV Parameters					
CD4 count (cells/μL)	75	35 [10, 76]	57	17 [8, 70]	0.16
HIV viral load ($\log_{10}$ copies/mL)	74	5.4 [5.1, 5.8]	57	5.5 [5.1, 5.7]	0.83
CSF Parameters [e]					
Opening pressure (mmH_2O)	59	260 [150, 360]	52	305 [215, 437]	0.05
Opening pressure > 250 mmH_2O, N (%)	59	32 (54%)	52	35 (67%)	0.16
Quantitative cryptococcal culture ($\log_{10}$ CFU/mL)	70	4.6 [2.9, 5.4]	53	5.4 [4.7, 5.8]	0.001
Cryptococcal antigen titer (1:x)	72	2000 [450, 7200]	55	4096 [1280, 12800]	0.01
White blood cells (/μL)	72	50 [<5, 145]	53	7 [<5, 31]	0.006
White blood cells < 5/μL	72	22 (31%)	53	25 (47%)	0.06

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Table 5.1 – continued from previous page

	Sterile at end of amphotericin		Not sterile at end of amphotericin		
	N with data	Median (IQR) [a]	N with data	Median (IQR) [a]	P-value [b]
Early fungicidal activity ($\log_{10}$ CFU/mL/day)	72	0.39 [0.33, 0.48]	57	0.24 [0.18, 0.30]	<0.001
Mortality					
3 weeks from end of amphotericin, N (%)	75	10 (13%)	57	13 (23%)	0.17
6 months from end of amphotericin, N (%)	75	20 (27%)	57	20 (35%)	0.34

[a] Medians and 25^{th} to 75^{th} percentile range (IQR, interquartile range) presented unless otherwise noted.

[b] P-values from chi-square test for frequencies and Wilcoxon ranksum test for medians. Fisher's exact test p-values reported for mortality comparison.

[c] Row percentages are presented.

[d] Column percentages are presented.

[e] CSF parameters from lumbar punctures performed at CM diagnosis.

Seventy-five individuals (57%) were found to have sterile CSF culture at the end of amphotericin therapy, while 57 (43%) had a positive culture. Individuals achieving a sterile CSF by the end of induction therapy were more likely to be female, had a slightly higher axillary body temperature, slightly lower hemoglobin, lower CSF opening pressure, lower fungal burdens in the CSF (by both quantitative culture and cryptococcal antigen titer), a higher rate of cryptococcal clearance, and were slightly more likely to have detectable white blood cells (>5 cells/μL) in the CSF at the time of CM diagnosis compared to individuals who did not achieve a sterile CSF culture (table 5.1).

Table 5.2: Baseline characteristics and outcomes by known and unknown sterility status at the end of amphotericin therapy for individuals with cryptococcal meningitis in the COAT trial.

	Known sterility status		**Unknown sterility status**		
N per group	132 (86%)		22 (14%)		
	N with data	Median (IQR) [a]	N with data	Median (IQR) [a]	P-value [b]
Study site, N (%) [c]	132		22		0.43
Kampala		82 (87%)		12 (13%)	
Mbarara		26 (79%)		7 (21%)	
Cape Town		24 (89%)		3 (11%)	
Treatment Group, N (%) [d]	132		22		0.51
Early ART		62 (47%)		12 (55%)	
Deferred ART		70 (53%)		10 (46%)	
Demographic and Clinical Parameters					
Age (years)	132	36 [29, 42]	22	34 [27, 38]	0.22
Males, N (%)	132	76 (58%)	22	11 (50%)	0.51
Weight (kg)	94	54.3 [46.0, 60.0]	18	54.0 [48.8, 59.3]	0.86
Weight missing, N (%)	132	38 (29%)	22	4 (18%)	0.30
Headache duration (days)	127	14 [7, 21]	22	12 [6, 30]	0.54
Karnofsky Score	131	50 [40, 50]	22	55 [40, 60]	0.12
Glasgow Coma Scale, N (%)	131		22		0.02
<15		35 (27%)		1 (5%)	
15		96 (73%)		21 (96%)	
Heart rate (beats per minute)	131	80 [69, 90]	22	77 [66, 83]	0.56
Respiratory rate (breaths per minute)	129	20 [20, 24]	22	20 [19, 22]	0.14
Systolic blood pressure (mmHg)	129	110 [108, 122]	22	109 [100, 117]	0.08
Diastolic blood pressure (mmHg)	129	70 [60, 82]	22	65 [60, 74]	0.06
Axillary temperature (^{o}C)	131	36.6 [36.0, 37.3]	21	36.8 [36.2, 37.5]	0.32
Fever (axillary temperature > 37.5^{o}C), N (%)	131	26 (20%)	21	5 (24%)	0.68

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Table 5.2 – continued from previous page

	Known sterility status		Unknown sterility status		
	N with data	Median (IQR) [a]	N with data	Median (IQR) [a]	P-value [b]
Clinical Laboratory Values					
Hemoglobin (g/dL)	129	11.5 [9.4, 13.0]	22	10.9 [9.4, 12.2]	0.62
Hematocrit (%)	129	34.0 [28.2, 38.5]	22	30.6 [27.3, 37.0]	0.46
White blood cells (x$10^3/\mu$L)	129	3.5 [2.5, 5.1]	22	3.4 [2.9, 5.2]	0.44
Creatinine (mg/dL)	128	0.7 [0.5, 0.9]	22	0.7 [0.5, 0.8]	0.89
Potassium (mmol/L)	116	3.9 [3.4, 4.3]	21	3.8 [3.5, 4.4]	0.64
HIV Parameters					
CD4 count (cells/μL)	132	21 [9, 72]	22	37 [13, 75]	0.70
HIV viral load ($\log_{10}$ copies/mL)	131	5.5 [5.1, 5.7]	22	5.8 [5.4, 5.9]	0.007
CSF Parameters [e]					
Opening pressure (mmH_2O)	111	280 [180, 382]	19	226 [170, 350]	0.31
Opening pressure > 250 mmH_2O, N (%)	111	67 (60%)	19	8 (42%)	0.14
Quantitative cryptococcal culture ($\log_{10}$ CFU/mL)	123	5.1 [3.8, 5.6]	22	5.4 [4.2, 5.8]	0.27
Cryptococcal antigen riter (1:x)	127	2560 [512, 8000]	22	2024 [1024, 16000]	0.41
White blood cells (/μL)	125	12 [<5, 105]	20	9 [<5, 62]	0.45
White blood cells < 5/μL, N (%)	125	47 (38%)	20	8 (40%)	0.84
Early fungicidal activity ($\log_{10}$ CFU/mL/day)	129	0.32 [0.23, 0.40]	16	0.36 [0.19, 0.44]	0.82
Mortality					
3 weeks from end of amphotericin, N (%)	132	23 (17%)	22	3 (14%)	>0.99
6 months from end of amphotericin, N (%)	132	40 (30%)	22	5 (23%)	0.61

[a] Medians and 25^{th} to 75^{th} percentile range (IQR, interquartile range) presented unless otherwise noted.

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Table 5.2 – continued from previous page

	Known sterility status		Unknown sterility status		
	N with data	Median (IQR) [a]	N with data	Median (IQR) [a]	P-value [b]

[b] P-values from chi-square test for frequencies and Wilcoxon ranksum test for medians. Fisher's exact test p-values reported for mortality comparison.

[c] Row percentages are presented.

[d] Column percentages are presented.

[e] CSF parameters from lumbar punctures performed at CM diagnosis.

Compared to those included in the primary analysis, the 22 individuals without a CSF culture result at the end of amphotericin therapy, who were excluded from the primary analysis because sterility status could not be ascertained, were more likely to have normal mental status at CM diagnosis (GCS of 15), marginally lower blood pressure, and had slightly higher HIV viral load, but otherwise had similar clinical and demographic characteristics as those included in the overall analysis (table 5.2).

5.3.2 Mortality Outcomes

Forty individuals (30%) died within 6 months of the end of amphotericin-based therapy (table 5.3). The median time to death, up to 6 months, was 20 days from the end of induction therapy (25^{th} to 75^{th} percentile range of 7 to 36 days). Over half, or 23 deaths, occurred in the first 3 weeks and the median time to death during this period was 7 days from the end of induction therapy (25^{th} to 75^{th} percentile range of 5 to 17 days).

When stratified by CSF sterility status at the end of amphotericin, 13% of those with a sterile CSF died within 3 weeks compared to 23% of those without a sterile culture (figure 5.2). In unadjusted analysis, sterility was not significantly associated with lower mortality, nor was there an association when COAT treatment arm and baseline fungal burden were

Table 5.3: Percent mortality by sterility status at the end of amphotericin therapy among individuals with cryptococcal meningitis in the COAT trial.

	Total	Died	Mortality Percent (95% CI)
3-week mortality			
Overall	132	23	17.4 [11.0, 23.9]
Sterile CSF	75	10	13.3 [5.6, 21.0]
Non-sterile CSF	57	13	22.8 [11.9, 33.7]
0-99 CFU/mL	28	6	21.4 [6.2, 36.6]
100-999 CFU/mL	17	3	17.6 [0.0, 35.8]
$\geq$ 1,000 CFU/mL	12	4	33.3 [6.7, 60.0]
6-month mortality			
Overall	132	40	30.3 [22.5, 38.1]
Sterile CSF	75	20	26.7 [16.7, 36.7]
Non-sterile CSF	57	20	35.1 [22.7, 47.5]
0-99 CFU/mL	28	9	32.1 [14.8, 49.4]
100-999 CFU/mL	17	7	41.2 [17.8, 64.6]
$\geq$ 1,000 CFU/mL	12	4	33.3 [6.7, 60.0]

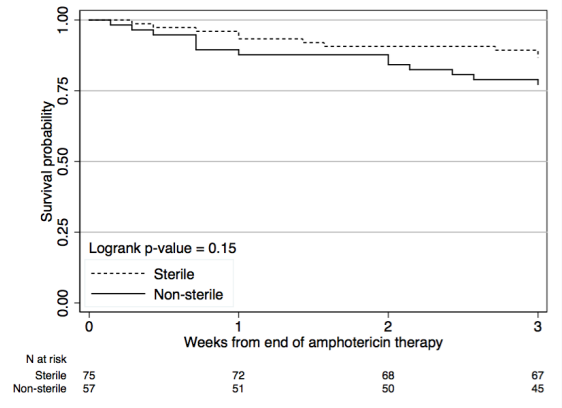

Figure 5.2: Three-week Kaplan-Meier survival probabilities by sterility status at the end of amphotericin therapy for individuals with cryptococcal meningitis in the COAT trial.

accounted for (table 5.4). Additionally, there did not appear to be an association with mortality when sterility was considered as a binary variable, a categorical variable, or when considering both a binary and continuous variable.

The differences in 6-month mortality by sterility status were even less prominent than

with the 3-week endpoint (figure 5.3). A total of 20 deaths (27%) were observed in those with a sterile CSF culture and 20 deaths (35%) were observed in those without a sterile culture. Adjusted estimates of the hazard ratios did not suggest that sterility was associated with 6-month mortality (table 5.5).

Table 5.4: Cox proportional hazard model results for the association of CSF sterility at the end of amphotericin therapy and 3-week mortality among individuals with cryptococcal meningitis in the COAT trial.

	Crude model	Adjusted model 1 [a]	Adjusted model 2 [b]
Binary variable			
Total subjects (deaths)	132 (23)	132 (23)	123 (22)
	HR (95% CI) [c]	HR (95% CI) [c]	HR (95% CI) [c]
Sterile	Ref	Ref	Ref
Non-sterile	1.82 [0.80, 4.14]	1.98 [0.86, 4.52]	1.45 [0.60, 3.48]
Binary + Continuous variable			
Total subjects (deaths)	132 (23)	132 (23)	123 (22)
	HR (95% CI)	HR (95% CI)	HR (95% CI)
Sterile	Ref	Ref	Ref
Non-sterile	1.18 [0.30, 4.59]	1.50 [0.37, 6.02]	1.04 [0.22, 4.95]
Continuous (per $\log_{10}$ CFU/mL increase)	1.21 [0.77, 1.90]	1.12 [0.71, 1.79]	1.15 [0.69, 1.92]
Categorical			
Total subjects (deaths)	132 (23)	132 (23)	123 (22)
	HR (95% CI)	HR (95% CI)	HR (95% CI)
Sterile	Ref	Ref	Ref
0-99 CFU/mL	1.74 [0.63, 4.78]	2.09 [0.75, 5.81]	1.63 [0.54, 4.95]
100-999 CFU/mL	1.33 [0.37, 4.84]	1.37 [0.38, 4.97]	0.87 [0.23, 3.29]
$\geq$1000 CFU/mL	2.76 [0.86, 8.80]	2.64 [0.82, 8.42]	2.14 [0.66, 6.94]

[a] Adjusted model 1 adjusted for treatment group.
[b] Adjusted model 2 adjusted for treatment group and CSF quantitative culture at CM diagnosis. Nine individuals were missing baseline quantitative culture and have been excluded from the analysis.
[c] HR: Hazard ratio; CI: Confidence interval

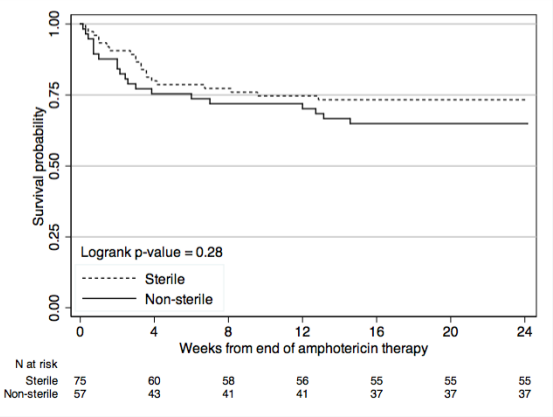

Figure 5.3: Six-month Kaplan-Meier survival probabilities by sterility status at the end of amphotericin therapy for individuals with cryptococcal meningitis in the COAT trial.

5.3.3 Mortality by Treatment Group

An exploratory subgroup analysis was conducted to investigate whether the association between CSF sterility and mortality was differential based on the timing of ART initiation. By 3 weeks after amphotericin therapy, those randomized to early ART had been on ART for 4 weeks, whereas those randomized to deferred ART had not yet begun ART. In the short-term, 3-week mortality was slightly higher among those receiving early ART (15%) compared to deferred ART (7%); however, the association of mortality with CSF sterility was similar between the treatment groups with a hazard ratio of 1.6 (95% CI: 0.6, 4.7) in the early ART group compared to 1.2 (95% CI: 0.3, 5.3) in the deferred ART group (p-value for interaction = 0.82, figure 5.4). No evidence for effect modification by treatment arm was seen with 6-month mortality, either (early ART HR = 1.1, 95% CI: 0.4, 2.6; deferred ART HR = 1.4, 95% CI: 0.5, 3.8; p-value for interaction = 0.69).

5.3.4 CM-related Mortality

The cause of death was available for all observed deaths. Twelve (52%) of the 23 deaths within 3 weeks and 14 (35%) of the 40 deaths within 6 months of the end of induction

Table 5.5: Cox proportional hazard model results for the association of CSF sterility at the end of amphotericin therapy and 6-month mortality among individuals with cryptococcal meningitis in the COAT trial.

	Crude model	Adjusted model 1 [a]	Adjusted model 2 [b]
Binary variable			
Total subjects (deaths)	132 (40)	132 (40)	123 (38)
	HR (95% CI) [c]	HR (95% CI) [c]	HR (95% CI) [c]
Sterile	Ref	Ref	Ref
Non-sterile	1.40 [0.75, 2.61]	1.47 [0.79, 2.74]	1.21 [0.62, 2.34]
Binary + Continuous variable			
Total subjects (deaths)	132 (40)	132 (40)	123 (38)
	HR (95% CI)	HR (95% CI)	HR (95% CI)
Sterile	Ref	Ref	Ref
Non-sterile	1.32 [0.48, 3.69]	1.52 [0.54, 4.31]	1.28 [0.41, 4.01]
Continuous (per $\log_{10}$ CFU/mL increase)	1.03 [0.70, 1.50]	0.98 [0.67, 1.44]	0.97 [0.64, 1.48]
Categorical			
Total subjects (deaths)	132 (40)	132 (40)	123 (38)
	HR (95% CI)	HR (95% CI)	HR (95% CI)
Sterile	Ref	Ref	Ref
0-99 CFU/mL	1.29 [0.59, 2.84]	1.43 [0.65, 3.17]	1.22 [0.52, 2.86]
100-999 CFU/mL	1.56 [0.66, 3.70]	1.58 [0.67, 3.74]	1.16 [0.47, 2.85]
≥1000 CFU/mL	1.42 [0.48, 4.15]	1.38 [0.47, 4.05]	1.27 [0.43, 3.77]

[a] Adjusted model 1 adjusted for treatment group.
[b] Adjusted model 2 adjusted for treatment group and CSF quantitative culture at CM diagnosis. Nine individuals were missing baseline quantitative culture and have been excluded from the analysis.
[c] HR: Hazard ratio; CI: Confidence interval

therapy were considered to be related to the initial CM episode. The CM-related 3-week mortality rate was slightly lower in individuals with a sterile CSF culture at the end of amphotericin (4% compared to 16% among those with a non-sterile culture). In crude analysis, this resulted in a hazard ratio of 4.2 (95% CI: 1.1, 15.4); however, after adjustment for COAT treatment arm and baseline fungal burden the hazard ratio was slightly attenuated and became non-significant (table 5.6). There was also a non-significant increase in CM-related mortality 6 months from the end of amphotericin therapy among individuals with a non-sterile CSF.

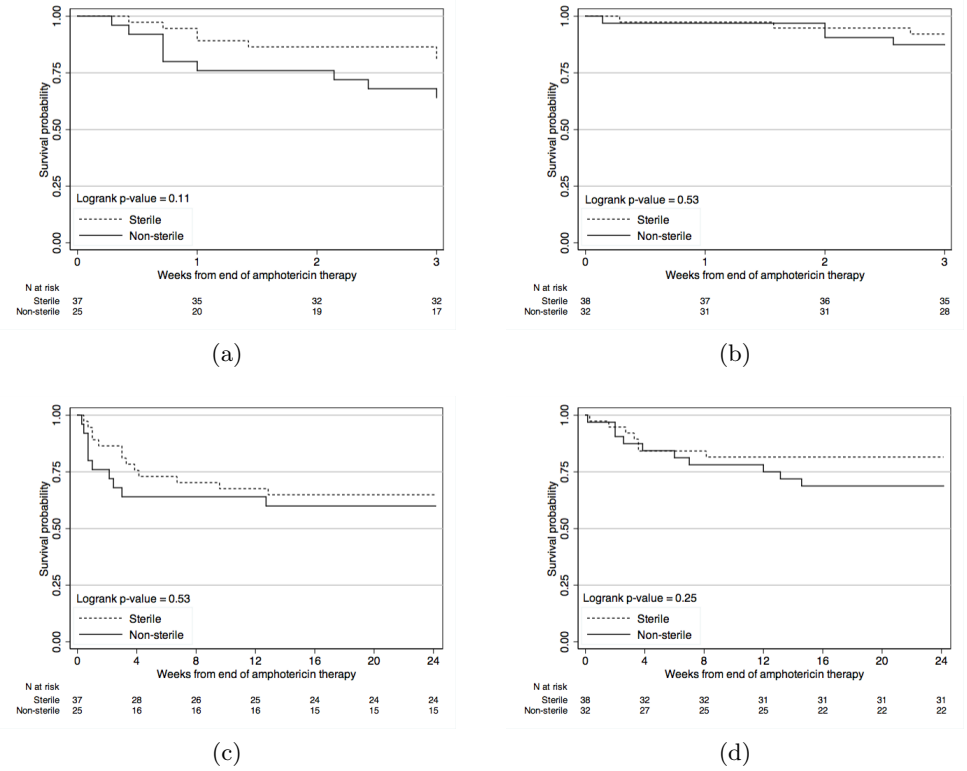

Figure 5.4: Kaplan-Meier survival probabilities by sterility status at the end of amphotericin therapy for (a) 3-week mortality in the early ART treatment arm, (b) 3-week mortality in the deferred ART treatment arm, (c) 6-month mortality in the early ART treatment arm, and (d) 6-month mortality in the deferred ART treatment arm for individuals with cryptococcal meningitis in the COAT trial.

5.3.5 Impact of Fluconazole

Individuals found to have a non-sterile culture at the end of amphotericin therapy were recommended to continue a higher dose of fluconazole beyond the 3 additional weeks described in the protocol. Therefore, the duration of fluconazole dosing was differential between the sterility groups and may have confounded the mortality association.

Looking at the full 6 months of follow-up, table 5.7 describes the distribution of individuals according to the time they switched to the lower, 400 mg/day, dose of oral fluconazole. Among those who had a non-sterile culture, a significantly higher proportion

Table 5.6: Adjusted cox proportional hazard model results for the association of CSF sterility at the end of amphotericin therapy and CM-related mortality among individuals with cryptococcal meningitis in the COAT trial [a].

	3-week CM-related Mortality	6-month CM-related Mortality
Binary variable		
Total subjects (deaths)	123 (11)	123 (13)
	HR (95% CI) [b]	HR (95% CI)
Sterile	Ref	Ref
Non-sterile	2.93 [0.74, 11.55]	2.61 [0.77, 8.88]
Binary + Continuous variable		
Total subjects (deaths)	123 (11)	123 (13)
	HR (95% CI)	HR (95% CI)
Sterile	Ref	Ref
Non-sterile	3.00 [0.36, 24.92]	2.22 [0.33, 14.81]
Continuous (per $\log_{10}$ CFU/mL increase)	0.99 [0.51, 1.93]	1.07 [0.59, 1.95]
Categorical		
Total subjects (deaths)	123 (11)	123 (13)
	HR (95% CI)	HR (95% CI)
Sterile	Ref	Ref
0-99 CFU/mL	3.23 [0.62, 16.89]	2.26 [0.48, 10.59]
100-999 CFU/mL	2.52 [0.48, 13.25]	2.92 [0.68, 12.47]
$\geq$1000 CFU/mL	3.22 [0.53, 19.58]	2.70 [0.48, 15.11]

[a] Model adjusted for treatment group and baseline CSF quantitative culture. Nine individuals were missing baseline CSF fungal burden so have been excluded from the models, including 1 individual with a CM-related death within 3 weeks and 1 more individual with a CM-related death within 6 months of the end of amphotericin therapy.
[b] HR: hazard ratio, CI: confidence interval

(37%) remained on a higher dose of fluconazole and switched to 400 mg/day after 3 weeks from the end of amphotericin therapy, as compared to 21% among those with a sterile CSF culture. None of the patients who died within 3 weeks of the end of amphotericin therapy were switched to the lower 400 mg/day fluconazole dose and only 8 of the 40 who died (20%) within 6 months of the end of amphotericin therapy were ever switched to the lower dose of 400 mg/day.

Adjusting for the dosage of fluconazole during follow-up, in addition to treatment group and baseline CSF fungal burden, did change the point estimate of the hazard ratio but did not alter the conclusions regarding the association between CSF sterility status and

Table 5.7: Distribution of switching from higher dose oral fluconazole to lower dose oral fluconazole after amphotericin therapy among individuals with cryptococcal meningitis in the COAT trial.

	Sterile at end of amphotericin		Not sterile at end of amphotericin		P-value [a]
	N with data		N with data		
N per group		75 (49%)		57 (37%)	
Switch to 400 mg/day fluconazole [b]	75		57		0.01
Died prior to switch, N (%)		15 (20%)		13 (23%)	
Switched before 3 weeks, N (%)		38 (51%)		13 (23%)	
Switched at 3 weeks, N (%)		6 (8%)		10 (18%)	
Switched after 3 weeks, N (%)		16 (21%)		21 (37%)	
Median time to switch (days from end of amphtericin)	59	23 [21, 45]	40	40 [22, 54]	0.04

[a] P-values from chi-square test of frequencies and Wilcoxon ranksum for medians.
[b] Protocol suggested switching to 400 mg/day fluconazole 3 weeks after the end of amphotericin. A one-week window was allowed around the 3 weeks. Patients with persistent culture positivity were recommended to switch to 400 mg/day after culture was confirmed to the negative.
[c] Protocol suggested switching to 200 mg/day fluconazole 11 weeks after the end of amphotericin. A one-week window was allowed around the 11 weeks.

6-month mortality (HR: 0.9, 95% CI: 0.5, 1.6).

5.3.6 Imputation of Sterility Status

Sensitivity analysis of the mortality associations was conducted using multiple imputation to estimate the CSF sterility status of individuals without a final CSF culture at the end of amphotericin therapy, as well as to account for missing baseline characteristics including baseline fungal burden. After multiple imputation, the hazard ratio for 3-week mortality, adjusted for treatment group and baseline fungal burden, was 1.3 (95% CI: 0.6, 3.1) and for 6-month mortality was 1.0 (95% CI: 0.6, 1.9) for the binary sterility categorization. The adjusted hazard ratios for CM-related mortality were also quite similar, but attenuated, after multiple imputation (3-week mortality HR: 2.3, 95% CI: 0.6, 8.9; 6-month mortality HR: 2.2, 95% CI: 0.7, 7.2).

5.4 Conclusions and Discussion

The objective of this analysis was to evaluate whether residual cryptococcal infection at the end of amphotericin-based induction therapy was associated with higher mortality within 3 weeks and 6 months. Of the 132 CM patients with HIV in the Cryptococcal Optimal ART Timing (COAT) trial who survived to the end of amphotericin therapy and had a final culture to assess sterility, 57% had a sterile CSF culture at that time. There was slightly lower 3-week mortality in those with a sterile culture; however, this decrease in mortality was not found to be statistically significant. Nor was there a significant difference in 6-month mortality seen between the sterility groups.

Several studies of CM patients have previously described the ramifications of residual viable cryptococcal cells after amphotericin-based therapy, noting that non-sterility is associated with persistently postive cultures later during consolidation therapy [25, 41] and increased risk of immune reconstitution inflammatory syndrome (IRIS) [43, 58]. Two further studies have examined the association between 6-month mortality and positive CSF cultures after CM induction therapy [43, 83]. In Thailand, where consolidation therapy consisted of 400mg/day fluconazole or 400 mg/day itraconazole, a 3.6-fold greater risk of death was observed in patients with non-sterile CSF (95% CI for the relative risk: 1.9, 6.4), of whom a staggeringly high proportion (64%) died within 6 months compared to just 18% mortality in those with sterile CSF [83]. A greater frequency of death at 6 months was also observed among patients with non-sterile CSF in a South African study, with 26% mortality in those with a non-sterile culture compared to 14% in those with a sterile culture, though this difference failed to reach statistical significance (HR: 1.9, 95% CI: 0.81, 4.6) [43]. The South African cohort was treated with 400 mg/day fluconazole for 8-12 weeks after induction therapy ended. A similar trend was observed in this study where 35% of those with a non-sterile culture died within 6 months compared to 27% in those

with a sterile culture. However, this difference was not statistically significant.

The current study additionally estimated that mortality 3 weeks after the end of amphotericin was roughly 17% overall, and only slight, non-significant differences were seen between those who had a sterile culture versus non-sterile culture. Few studies have remarked on the impact of residual fungal burden and shorter-term mortality, even though mortality soon after induction therapy remains high. Robinson, and colleagues, found that 19% of those with a non-sterile CSF culture died within 8 weeks of the end of amphotericin therapy, compared to just 12% of those with a sterile culture [41]. Examination of the survival curve from the recent South African study suggests that mortality was very similar by sterility status through the first month after amphotericin therapy and did not begin to diverge until after this period [43]. Therefore, in the presence of consolidation therapy there does not appear to be a very large impact of incomplete clearance of cryptococcus on short-term mortality.

Several possible reasons exist for why this analysis did not identify an association with mortality. First, high dose fluconazole (800 mg/day) was continued for 3 weeks following the end of amphotiericin in this study. This dosage is known to continually kill *C. neoformans* and, thus, may have quickly eliminated any remaining fungus that could have an impact on short- and long-term mortality risk. A phase II clinical trial conducted in Thailand and the US demonstrated a trend toward lower 6-month mortality with a treatment regimen that continued 800mg/day fluconazole for 3 weeks into the consolidation phase compared to regimens that did not contain fluconazole and a regimen that contained 400 mg/day fluconazole [100]. All other studies assessing the association of CSF sterility with mortality have used a lower doseage of fluconazole, or itraconazole, for consolidation therapy and, therefore, the higher dose in this study may have contributed to a null finding.

Secondly, the relatively small size of this cohort may have limited statistical power. Relatedly, the patients included in this analysis were participating in a clinical trial and,

as such, may have experienced lower mortality than an observational cohort of patients unaffiliated with a trial. Mortality 6 months after the end of amphotericin therapy was 30% in this cohort, which is certainly less than the mortality observed in clinical cohorts described by Pitisuttithum, et al. [83], in Thailand, but slightly higher than was observed by Chang, et al. [43]. Indeed, Chang and colleagues may have also faced limited power in their analysis for 6-month mortality.

A third reason why an association may not have been identified is that all patients were started on antiretroviral therapy, which may have augmented or supported recovery from CM through restoration of immune function. The two studies prior to 2000 did not mention ART use before or after CM therapy, so it is unclear whether ART played a role in the observed mortality associations [41, 83]. It is possible that only a detrimental effect of residual fungal burden would be seen in patients who do not initiate HIV therapy after CM therapy. While not the goal of the current analysis, this hypothesis could be vaguely addressed with the subgroup analysis by COAT trial arm. By the 3-week mortality endpoint, 48% of the cohort had initiated ART, in accordance with their randomization assignment. In the group that had not initiated ART there was little to no difference in the mortality proportions or the Kaplan-Meier survival estimates by sterility status. In fact, any signal of a detrimental effect of residual fungal burden in this cohort was seen only among patients who initiated ART prior to the end of CM induction therapy.

Another possible reason for a null association is that all-cause mortality was used in the main primary analysis. The severity of advanced AIDS leaves many CM patients vulnerable to concomitant opportunistic infections that are also related to increased risk of death. Thus, it is possible that overall mortality is universally high among all patients post-CM, and CSF sterility status after induction therapy has very little contribution to the risk of overall mortality. In this cohort of patients, 52% of the deaths soon after induction therapy were deemed to be due to CM. Furthermore, only 2 additional deaths related to the

initial CM episode were identified after the 3-week endpoint, suggesting that the majority of CM-related deaths occur shortly after induction therapy ends. When considering only CM-related deaths in this analysis, the point estimates for the risk of death were greater than in the all-cause mortality analysis; however, the associations remained non-significant, after covariate adjustment. Because many of the deaths after 3 weeks appeared to be due to causes other than CM, future studies investigating factors contributing to CM-related mortality should consider restricting analysis to a shorter time period than 6 months in order to get more accurate estimates.

In conclusion, this analysis suggests that, with high levels of clinical care during induction therapy and with a consolidation regimen including high dose fluconazole, residual fungal infection in the CSF at the end of induction therapy for HIV-associated CM was not found to contribute substantially to increased 3-week or 6-month mortality.

5.5 Exploratory Analysis

5.5.1 EFA Association with Mortality

A strong correlate of CSF sterility at the end of amphotericin-based therapy is the early fungicidal activity (EFA), or the rate of fungal clearance. Mortality after induction therapy was unassociated with EFA during amphotericin therapy in this cohort. Among those who died within 3 weeks of the end of amphotericin, the median EFA was 0.31 CFU/mL/day (25^{th} to 75^{th} percentile: 0.22, 0.39 CFU/mL/day) compared to 0.34 CFU/mL/day (25^{th} to 75^{th} percentile: 0.23, 0.41 CFU/mL/day) among those who survived this period (p-value = 0.77). The median EFA for those who died with 6 months of the end of amphotericin (0.32 CFU/mL/day, 25^{th} to 75^{th} percentile: 0.22, 0.39 CFU/mL/day) was similar to those who survived (0.34 CFU/mL/day, 25^{th} to 75^{th} percentile: 0.23, 0.43 CFU/mL/day; p-value = 0.58). After adjustment in Cox regression there was also no evidence of an association

between EFA with mortality (3-week mortality: HR = 0.8, 95% CI: 0.2, 3.7; 6-month mortality: HR = 0.5, 95% CI: 0.1, 2.4).

Chapter 6

Conclusions

6.1 Discussion

Cryptococcal meningitis (CM) is a wide-spread, yet under-recognized, fungal opportunistic infection occurring primarily among people living with advanced HIV/AIDS. While vast advances in understanding the pathogenesis and treatment options for CM have reduced mortality, major gaps remain in understanding factors that contribute to the remaining mortality. The intent of this dissertation was to contribute towards the efforts to address these gaps and provide evidence that could further improve short and long-term recovery from HIV-associated CM in sub-Saharan Africa.

6.2 Summary of Findings and Implications

6.2.1 Chapter 3: Therapeutic lumbar punctures and acute mortality from cryptococcal meningitis

Chapter 3 was focused on the days after CM diagnosis and understanding the effect of lumbar punctures (LPs) on CM mortality. Raised intracranial pressure is common among

individuals presenting with CM and contributes to many of the disease's signs and symptoms. The most common approach to reducing intracranial pressure is an LP and, though based on indirect evidence of a link between high intracranial pressure and CM mortality, current treatment guidelines stress the importance of repeated LPs over the course of therapy to reduce pressure. However, no direct estimates of the effect of therapeutic lumbar punctures on mortality are available in the CM literature. The primary objective of Chapter 3 was to estimate the effect of therapeutic LPs on mortality within 11 days of CM diagnosis.

The current analysis adds direct supportive evidence that at least one repeat LP during the first 11 days of CM induction therapy reduces 11-day mortality by 69% (95% CI: 18% to 88%), adjusted for baseline heart rate, CSF fungal burden, and altered mental status. This beneficial effect was independent of the baseline CSF opening pressure, demonstrating that increases in intracranial pressure may be common among all CM patients and that all patients may benefit from an additional LP. As discussed in Chapter 3, future research is warranted to confirm these results in a broader population of HIV-positive individuals with CM. The best approach for implementation of therapeutic LPs during CM therapy should also be investigated, namely to inform when additional LPs should be conducted and whether LPs should succeed symptoms or be systematically applied.

6.2.2 Chapter 4: Predictive factors of CSF sterility at the end of amphotericin -based therapy for cryptococcal meningitis

Still focused on the acute, 2-week period after CM diagnosis, the objective of Chapter 4 was to investigate baseline demographic and clinical features that are predictive of treatment success. The recommended treatment regimen for CM consists of 2 weeks of amphotericin-based induction therapy to rapidly remove infection from the central nervous system. Despite antifungal effects of amphotericin, nearly one half of CM patients will continue to

have viable fungus in their central nervous system at the end of induction therapy. Few cohorts from sub-Saharan Africa have described factors predictive of patients who will and will not achieve a sterile culture. Being able to predict patient outcomes has many advantages, including the potential for customizing therapy. The findings from this chapter could be useful in devising strategies for customized CM therapy.

Analysis of the treatment outcomes in CM patients enrolled in the Cryptococcal Optimal ART Timing (COAT) trial, in Chapter 4, demonstrated that the baseline CSF quantitative fungal burden was a strong and practical predictor of achieving CSF sterility after 2 weeks of amphotericin-based therapy. Information on the burden of infection could possibly be used to tailor the duration of amphotericin, thus avoiding unnecessary toxicity and treatment costs for individuals with a lower burden of infection and potentially shifting resources to allow for more aggressive treatment of high-risk patients. For example, patients with high levels of infection could be identified as candidates for adjunctive therapy, such as with flucytosine [25, 85] or interferon-γ [58]. Future research could build off evidence presented in this dissertation and investigate the feasibility and efficacy of customized treatment on CM outcomes.

6.2.3 Chapter 5: Effect of CSF sterility at the end of amphotericin-based therapy for cryptococcal meningitis on subsequent 3-week and 6-month mortality

The final chapter, Chapter 5, was focused on understanding the consequences of residual fungal infection after induction therapy and, particularly, the implications for mortality in the first weeks and months after induction therapy ends. Mortality after CM diagnosis is markedly high during the first 2 weeks and remains fairly rapid and consistent through the first month after induction therapy ends, at which point the risk of death appears

to taper off. Previous analyses of cohorts, primarily before the widespread use of highly active antiretroviral therapy (HAART) for HIV, have suggested that a non-sterile culture at the end of induction therapy is associated with higher mortality within 6 months. More contemporary studies in sub-Saharan Africa have found similar, though non-significant, trends towards higher mortalty within 6 months for patients with persistently postive CSF cultures, however no studies have investigated the role that residual infection plays on mortality in the first 3 weeks after induction therapy. The objective of Chapter 5 was to evaluate the impact of residual infection on 3-week mortality and to also provide further evidence for the effect of residual infection on 6-month mortality.

Among HIV-positive individuals in the COAT trial surviving the 2-week phase of amphotericin-based induction therapy, there was no evidence that either the presence or the amount of residual cryptococcal infection in the CSF had an association with moratlity in the following 3 weeks. Additionally, there was no evidence that residual infection was associated with mortality within 6 months of the end of amphotericin therapy. While unable to directly test this hypothesis, it is possible that the dose of fluconazole used at the end of amphotericin therapy may have contributed to the lack of association. Most prior cohorts have used a lower dose of fluconazole during consolidation therapy, which may have inadequately cleared the remaining infection and supported the association between residual infection and mortality. Several other reasons for a null association were explored in the discussion section, namely a small sample size and the impact of antiretroviral therapy (ART). Understanding the role that residual infection may play in the recovery from CM has vast implications for research into more potent antifungal therapy. Therefore, future studies should evaluate larger cohorts and investigate the effect of higher dose fluconazole during consolidation therapy on CM recovery.

6.3 Limitations

As with all observational studies, the analyses conducted as part of this dissertation include several assumptions, which, if not met, preclude causal interpretations of the described associations and inference to the wider population of HIV-positive individuals with CM. The underlying assumptions are that: 1) the study sample is representative of the study population of interest, 2) measurements of the exposures, clinical characteristics, and outcomes were valid and accurate in the entire study sample, 3) the comparison groups considered in each chapter were similar with regards to all other aspects beyond the exposure being compared, and 4) the models constructed are correctly specified for the study population, the data collected, and the questions of interest. Each chapter discussed the relevance of many of these assumptions in the context of the question at hand. However, several common limitations throughout this dissertation warrant brief discussion.

One common limitation is the limited sample size from which to draw epidemiologic and clinical conclusions. As only one source of data was used throughout, the sample size of all three chapters was limited by the size of the COAT trial. Very little can be done to augment statistical power while working within the confines of a single study population. Thus, caution was taken to ensure that conclusions regarding null findings considered the sample size and power limitations.

Furthermore, the use of a single data source limits the external validity, or generalizability, of the conclusions. The majority of the CM patients included in the COAT trial presented to care in Kampala, Uganda, with roughly one third of patients coming from external sites in Mbarara, Uganda and Cape Town, South Africa. The treatment experience for patients with CM presenting to COAT study sites are likely reflective of patients presenting to tertiary teaching hospitals, similar to Mulago Hospital in Kampala, in other

metropolitan cities of sub-Saharan Africa, but are much less reflective of patients presenting with meningitis to primary or district hospitals in more rural areas of sub-Saharan Africa. This limitation may temper the validity of the mortality rates and the rates of CSF sterility to many other settings where access to essential medicines, such as amphotericin and ART, and essential services, such as laboratory diagnostics, nursing care, and supplies for lumbar punctures, are more limited. However, the treatment outcomes observed in the combined cohort from Kampala, Mbarara, and Cape Town were quite similar to the outcomes reported in the literature from patients in the US, Thailand, France, and rural South Africa, supporting the validity of the analyses in this dissertation to other tertiary care facilities that routinely encounter CM.

Finally, patients included in this dissertation were drawn from patients screened for and enrolled in a clinical trial. As such, the patients may be different from patients in an observational clinical cohort and the care received as part of the trial may have differed from the care received in a non-trial setting. Both of these differences further contribute to limitations of the external validity of the findings. Additionally, the clinical trial setting may have contributed to the limited sample size because of lower observed mortality, as discussed in Chapter 5. In contrast, however, enrolled patients in the COAT trial may have been quite similar to the underlying patient population with meningitis at each of the trial sites, as very few patients screened for enrollment refused randomization (Chapter 3, figure 3.1).

6.4 Strengths

Despite these limitations, many strengths of these analyses exist and augment their implications. First, while clinical trials can be limited in terms of generalizability to different settings, trials are an immense source of rich, high-quality data. Data from the

COAT trial were quite complete and included many different clinical characteristics that have not been previously considered. Missing data was identified for each of the analyses included; however, sensitivity analyses using multiple imputation indicated that the conclusions throughout each chapter were robust to missing variables.

Sub-Saharan Africa has not only the greatest burden of HIV-positive individuals, but also has the highest occurrence of cryptococcal meningitis in the world. Therefore, the focus of the dissertation was on sub-Saharan Africa where the impact of any conclusions could be the greatest. Data from Cape Town provided information of CM treatment and recovery in a well-resourced setting, whereas data from Uganda provided information on CM outcomes in both a more rural tertiary care setting in Mbarara and a large referral teaching hospital in Kampala. This diversity enables the conclusions to be more broadly applicable. Furthermore, the implications of each chapter's findings were considered and discussed in light of the resource limitations that are common throughout sub-Saharan Africa.

Similarly, another strength of this dissertation is that it directly addressed gaps in the literature by focusing on questions that remain unanswered for CM treatment and recovery. Few studies have rigorously assessed epidemiologic associations of therapeutic lumbar punctures and residual cryptococcal infection with CM outcomes. This research collectively infoms our understanding of the current treatment guidelines, which were based largely on clinical observation and not on epidemiologic evidence, and provides evidence upon which future research and clinical practice can be shaped.

6.5 Conclusions

Cryptococcal meningitis is a costly consequence of advanced immunosuppression. Great advances have been made in CM treatment resulting in improved survival and reduced

morbidity. The objective of this dissertation was to extend the discussion about what the toolbox for CM treatment should contain in resource-limited settings. The results suggest that additional benefits could be gained from the use of therapeutic lumbar punctures during the acute phase of treatment, the possibility of customizing therapy to further reduce treatment toxicities, and, finally, describing the relationship between residual infection and CM mortality with indirect support for higher doses of fluconazole to be used early during the consolidation phase of therapy.

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Appendix A

Appendix for Chapter 3

A.1 Acute mortality association in analysis cohort without exclusions

The analysis of Chapter 3 was restricted to 248 subjects who were alive and had not already had a therapeutic lumbar puncutre (LP), died, or were censored the day after CM was diagnosed. These conditions excluded nine individuals from the primary analysis. Sensitivity analysis was conducted to assess whether the estimated effect of therapeutic LPs on acute mortality was robust to the exclusion of these nine individuals. Table A.1 presents the relative risks of acute mortality after, compared to before, a therapeutic LP from the marginal structural models and unweighted conventional Poisson regression models. The point estimates indicate that the model results presented in the main text of Chapter 3 were robust to exclusion of the nine individuals.

Table A.1: Estimated relative risk of acute mortality among those receiving a therapeutic lumbar puncture within 11 days of diagnosis of cryptococcal meningitis in HIV-positive, ART-naïve patients in South Africa and Uganda; including nine subjects excluded on the day of screening.

	Relative Risk	95% CI
Crude model	0.51	(0.20, 1.33)
Marginal Structural Model Pooled Poisson Regression [a]		
Adjusted model 1	0.48	(0.18, 1.27)
Adjusted model 2	0.37	(0.14, 1.02)
Adjusted model 3	0.29	(0.11, 0.77)
Adjusted model 4	0.30	(0.10, 0.85)
Fully adjusted model [b]	0.32	(0.10, 1.04)
Unweighted Pooled Poisson Regression		
Adjusted model 1	0.61	(0.23, 1.61)
Adjusted model 2	0.49	(0.18, 1.31)
Adjusted model 3	0.45	(0.17, 1.20)
Adjusted model 4	0.46	(0.17, 1.23)
Fully adjusted model [b]	0.46	(0.17, 1.27)

Confidence interval (CI); Glasgow Coma Score (GCS).
[a] Adjusted model 1 adjusted for heart rate.
Adjusted model 2 is adjusted model 1 additionally adjusted for CSF fungal burden.
Adjusted model 3 is adjusted model 2 additionally adjusted with an indicator for GCS <15.
Adjusted model 4 is adjusted model 3 additionally adjusted for CSF closing pressure.
[b] Fully adjusted model accounts for heart rate, CSF fungal burden, CSF closing pressure, indicator for GCS <15, and weight.

A.2 Baseline characteristics and outcomes of subjects with missing opening pressure at baseline

The opening pressure during the diagnostic lumbar puncture was commonly missing among the cohort of subjects included in the analysis of Chapter 3. Table A.2 presents a comparison of baseline characteristics and outcomes between individuals with measured and missing opening pressure. This comparison indicates that the two sets of patients were similar in many regards. One, perhaps expected, difference is that patients who had measured opening pressure had more CSF removed during the diagnostic LP compared to those who did not have measured opening pressure (p-value < 0.001).

Table A.2: Baseline characteristics and mortality by measurement of CSF opening pressure at diagnosis of cryptococcal meningitis among HIV-positive, ART-naïve individuals in South Africa and Uganda. [a]

	N with data	**Missing opening pressure**	N with data	**Measured opening pressure**	**P-value** [b]
N per group		40		208	
Site [c]	40		208		0.002
Kampala		21 (12%)		160 (88%)	
Mbarara		13 (34%)		25 (66%)	
Cape Town		6 (21%)		23 (79%)	
Age (years)	40	37 [29, 42]	208	35 [30, 40]	0.44
Males, N (%)	40	23 (58%)	208	112 (54%)	0.67
Weight (kg)	29	54.0 [43.2, 58.3]	135	53.0 [46.0, 59.3]	0.78
Missing weight	40	11 (28%)	208	73 (35%)	0.35
Headache duration (days)	37	14 [7, 21]	204	14 [7, 28]	0.20
Papilledema, N (%)	37	1 (3%)	197	7 (4%)	0.79
Karnofsky Score	39	50 [40, 60]	208	50 [40, 50]	0.42
Glasgow Coma Scale, N (%)	39		208		0.49
< 15		13 (33%)		58 (28%)	
15		26 (67%)		150 (72%)	
Heart rate (beats per minute)	39	84 [73, 97]	207	80 [69, 93]	0.11
Respiratory rate (breaths per minute)	38	20 [20, 24]	205	22 [20, 24]	0.31
Systolic blood pressure (mmHg)	39	110 [101, 125]	204	112 [106, 122]	0.60
Diastolic blood pressure (mmHg)	39	70 [60, 81]	204	70 [61, 81]	0.73
Axillary temperature (^{o}C)	39	36.9 [36.2, 37.9]	206	36.4 [35.9, 37.1]	0.01
Fever (axillary temperature $> 37.5^o$ C)	39	12 (31%)	206	37 (18%)	0.07
Clinical Laboratory Values					
Hemoglobin (g/dL)	35	10.9 [9.8, 12.1]	191	11.2 [9.2, 13.2]	0.56

Continued on next page

Table A.2 – continued from previous page

	N with data	**Missing opening pressure**	N with data	**Measured opening pressure**	**P-value** [b]
Hematocrit (%)	35	32.8 [28.3, 35.7]	191	33.2 [27.2, 39.0]	0.65
White blood cells (x$10^3/\mu$L)	35	3.4 [2.5, 5.7]	191	3.4 [2.6, 5.2]	0.93
Creatinine (mg/dL)	37	0.8 [0.6, 1.0]	196	0.7 [0.5, 0.9]	0.08
Potassium (mmol/L)	36	4.1 [3.7, 4.4]	183	3.9 [3.5, 4.3]	0.26
CSF Parameters					
Closing pressure (mmH_2O)	1	70 [70, 70]	189	90 [65, 125]	0.49
Amount of CSF removed during lumbar puncture (mL)	34	8 [5, 15]	206	16 [10, 25]	<0.001
Quantitative cryptococcal culture ($\log_{10}$ CFU/mL)	35	5.0 [3.9, 5.6]	199	5.2 [4.2, 5.6]	0.55
White blood cells (/μL)	37	5 [<5, 45]	195	10 [<5, 105]	0.14
White blood cells < 5 cells/μL	37	17 (46%)	195	83 (43%)	0.70
Outcome					
Died, N(%)	40	9 (23%)	208	27 (13%)	

[a] Medians [25^{th} - 75^{th} percentile], unless otherwise noted. Percentages are column percentages, unless otherwise noted.

[b] P-values from chi-square test for frequencies and ranksum test for medians.

[b] Row percentages are presented.

Appendix B

Appendix for Chapter 4

B.1 Early fungicidal activity calculations

The concept of quantifying the rate of microbiologic decline while on treatment has been described for many other infectious diseases, for example tuberculosis (where the rate of decline is known as the early bactericidal activity [101]) and malaria (where the rate is known the parasite clearance curve [102]). The objective of estimating the rate of cryptococcal clearance, also called the early fungicidal activity (EFA), in cryptococcal meningitis (CM) is to provide a quantitative metric for the decline in *C. neoformans* over the course of induction therapy. Efforts are being pursued to explore the predictive potential of the EFA for use in clinical practice and, potentially, as a surrogate marker in clinical trials of new and existing CM treatment regimens.

As initially described [29], the EFA estimates a person-specific slope of the association between the quantitative CSF fungal culture and the days on treatment. The slope estimates the decline in colony-forming units (CFU) of *C. neoformans* per mL of CSF per day, and, as the rate of decline appears to be exponential, quantitative culture is typically $\log_{10}$ transformed to estimate the decline in $\log_{10}$CFU/mL/day (see figure B.1). Quantitative

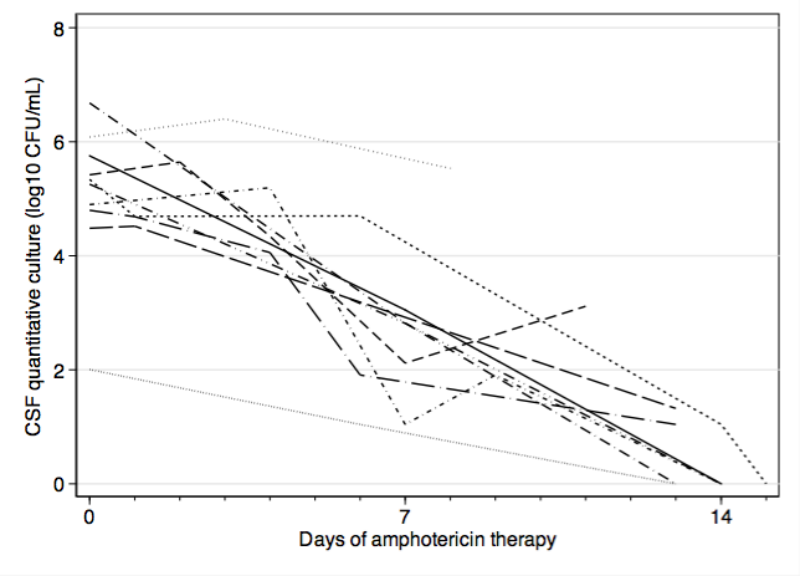


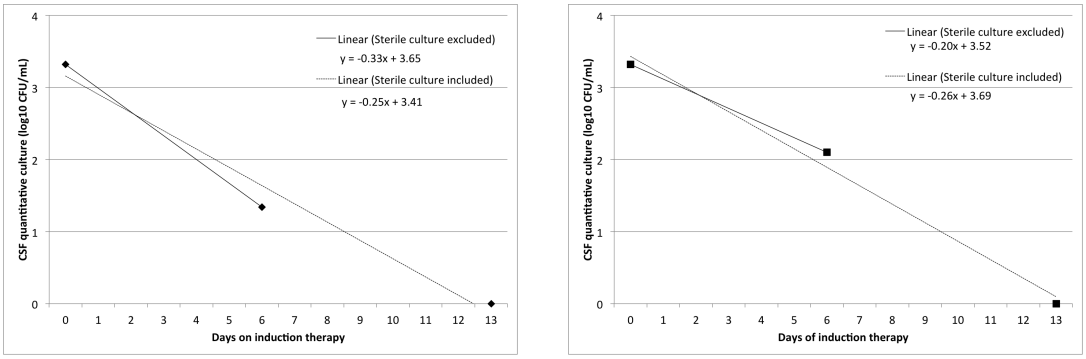
Figure B.1: Early fungicidal activity for a subset of HIV-positive individuals with cryptococcal meningitis in the COAT trial.

cultures are typically conducted just prior to the start of, mid-way through, and at the end of induction therapy. As induction therapy is generally 2 weeks, lumbar punctures (LPs) at CM diagnosis and at day 7 and 14 of therapy are typcially conducted to monitor quantitative cultures. Research groups have varied the timing of these prescribed lumbar punctures [60, 85] and many patients may also recieve additional LPs in order to control intracranial pressure. Therefore, the exact number of quantitative cultures available to calculate the EFA can differ by subject and research group.

Various methods exist for calculating the EFA, but, historically, the EFA is estimated from a subject-specific linear regression equation with $\log_{10}$ CFU/mL as the dependent variable and days of induction therapy as the independent therapy. The regression equation includes all available cultures during the first week of therapy. All cultures during the second week of therapy are also considered, however if the culture on day 14 is negative, then this culture is excluded from calculation if its inclusion makes the slope estimate

less steep [29]. This selective approach to including sterile cultures at the end of therapy combats possible underestimation of the slope when sterility is interval censored, meaning the exact timing of sterility is unknown but it is known to have occurred in the prior observation interval. It is unclear from the methods sections of papers calculating EFA whether sterile cultures observed during the first week of therapy are included or excluded from EFA estimation.

A slightly different approach has been taken in this dissertation and all cultures up to and including the first sterile culture were included, even if the first sterile culture was observed during the second week of induction therapy. This approach was chosen because it was recognized that inclusion of sterile cultures could either underestimate or overestimate the "true" EFA (see figure B.2) and exclusion of sterile cultures in the second week of therapy often restricted the number of cultures to 1 or 2, which reduces the accuracy of the EFA estimate. Apart from this departure, the EFAs described in this dissertation were calculated in a similar way to the historical approach using subject-specific linear regression equations.



(a) (b)

Figure B.2: Simulated EFA estimates comparing inclusion versus exclusion of sterile cultures at end of induction therapy. (a) Example where inclusion of sterile culture at end of induction therapy underestimates the EFA, and (b) example where inclusion of sterile culture at end of induction therapy overestimates the EFA.

The EFA used in analysis for Chapter 4 and Chapter 5 was equal to -1 times the regression slope in order to ease interpretation. Thus, a higher positive number indicates a more steep decline in CFU/mL/day.

B.2 Alternative definition of CSF sterility

In Chapter 4 the outcome of interest was sterility of the CSF at the end of 2 weeks of amphotericin, plus fluconazole, induction therapy for cryptococcal meningitis (CM). Sterility was defined as observation of a negative culture, with no subsequent positive cultures, before the end of induction therapy. An alternative definition of the outcome could have been used, and one possibility was to define the outcome in terms of a low, but non-zero, quantitative culture. Because amphotericin has a long half-life and because patients in the Cryptococcal Optimal ART Timing (COAT) trial were concurrently receiving high doses of fluconazole, another antifungal agent, individuals with a low quantitative culture burden at the end of induction therapy may have reached CSF sterility soon after the end of induction therapy.

A sensitivity analysis was conducted defining the outcome as no more than 100 colony-forming units (CFU)/mL on fungal culture by the end of induction therapy to assess the robustness of the results to outcome misclassification. Table B.1 presents the estimated adjusted odds ratio of reaching 100 CFU/mL or lower for changes in the clinical covariates found to be independently associated with the sterility outcome. Using this higher fungal burden threshold, the baseline quantitative fungal burden, from fungal culture, and the 1-week estimated early fungicidal activity (EFA) were both independently associated with a low fungal burden at the end of therapy. The cryptococcal antigen (CRAG) titer and the hemoglobin level at baseline were only marginally associated with a low fungal burden.

Table B.1: Multivariable logistic regression associations with CSF quantitative culture less than or equal to 100 CFU/mL by the end of amphotericin therapy among individuals with cryptococcal meningitis in the COAT trial.

Number reaching sterility [a]	117 (80%)
Number not reaching sterility (%)	29 (20%)
	OR (95% CI)[b]
CSF quantitative cryptococcal culture ($\log_{10}$ CFU/mL)	0.24 (0.10, 0.59)
Early fungicidal activity, day 0 to 11 (x10 $\log_{10}$ CFU/mL/day)	2.04 (1.40, 2.98)
Hemoglobin (g/dL)	0.80 (0.59, 1.08)
CSF cryptococcal antigen titer ($\log_2$ titer)	0.81 (0.64, 1.04)
Early fungicidal activity, day 0 to 11 (x10 $\log_{10}$ CFU/mL/day)	1.75 (1.27, 2.42)
Hemoglobin (g/dL)	0.80 (0.63, 1.03)

[a] Sterility was defined as any quantitative culture value of 100 CFU/mL or less.
[b] Odds ratio (OR) and 95% confidence intervals (CI).

Appendix C

Appendix for Chapter 5

C.1 Tests of proportional hazards assumption

Analysis of the association between CSF sterility at the end of amphotericin-based induction therapy and 3-week and 6-month mortality, in Chapter 5, was conducted using Cox proportional hazards regression. One assumption of this analysis approach is that the ratio of the hazard of the outcome, here 3-week mortality and 6-month mortality, is proportional over time between those with sterile CSF compared to non-sterile CSF. Table C.1 presents the test of this assumption in the models used throughout Chapter 5. In all cases, the non-significant p-value indicates that the proportional hazards assumption is suitable.

Table C.1: Tests of proportional hazards for Cox proportional hazard model results of the association of CSF sterility at the end of amphotericin therapy with 3-week and 6-month mortality among individuals with cryptococcal meningitis in the COAT trial.

	P-value [a]
3-week Mortality	
Crude [b]	0.73
Adjusted model 1 [c]	0.95
Adjusted model 2 [d]	0.58
6-month Mortality	
Crude [b]	0.76
Adjusted model 1 [c]	0.47
Adjusted model 2 [d]	0.56

[a] P-value for test of interaction between model covariates and follow-up time.
[b] Model includes only binary indicator for sterility status.
[c] Crude model adjusted for COAT treatment group.
[d] Crude model adjusted for COAT treatment group and baseline quantitative culture.